

REGULATION OF THE IMMUNE SYSTEM BY SYNTHETIC POLYNUCLEOTIDES

II. ACTION ON PERITONEAL EXUDATE CELLS*

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Knowledge of the cells potentiated by adjuvants is of importance in understanding their mode of action and in aiding delineation of the early, subliminal events in the processes leading to humoral and cell-mediated immunity. Laboratory models for initiation of antibody synthesis incorporate the sequential action of several types of cells (1-3). A speculative role for the macrophage as initiator of this sequence is postulated inasmuch as exposure of antigen to peritoneal exudate cells (PEC)¹ before injection can either initiate or enhance such systems (1, 4-8). Although immunogenic activity was found to be present in ribonucleic acid-rich fractions from such peritoneal cells, a question exists as to whether antibody synthesis resulted from trace antigen combined with a nucleic acid structure as a powerful adjuvant (9), or whether the nucleic acid was an induced mediator (10), or both were functional. Since defined homoribopolynucleotide complexes such as polyadenylic-polyuridylic acid (poly A:U) have been shown recently to act equally as well as nucleic acids as adjuvants in the stimulation of antibody synthesis *in vivo*² (11, 12), their capacity to increase the responsiveness of PEC exposed to antigen *in vitro* was investigated. The

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¹ *Abbreviations used in this paper:* BGG, bovine gamma globulin; BME, Eagle's basal medium; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; poly A, polyadenylic acid; poly A:U, polyadenylic-polyuridylic acid complex; poly I:C, polyinosinic-polycytidylic acid complex; poly U, polyuridylic acid; RFC, rosette-forming cells; SRBC, sheep red blood cells.

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conclusion that the PEC may be one of the cells affected by the homoribopolymers is supported by the data recorded herein. In addition, the capability of ribonucleic acid-rich fractions extracted from PEC to convey immunity under poly A:U stimulus is documented.

Materials and Methods

General.—Polyadenylic acid, potassium salt, (poly A, lot No. A-16744; 1104748) and polyuridylic acid, ammonium salt, (poly U, lot No. 45750; 411754) were purchased from Miles Laboratories, Inc., Elkhart, Ind. The polymers were prepared in a concentration of 3.0 mg/ml in 0.15 M NaCl and frozen separately at -20°C . These stock solutions were diluted 1:2 by mixing with each other to form the helical polymer (poly A:U) and allowed to stand at 4°C for 30 min before use. A final concentration of 150 $\mu\text{g}/\text{ml}$ of each was used unless otherwise indicated. Oligonucleotides of adenylic acid, A_3 – A_6 (Lots 12-5-392) also were obtained from Miles Chemical Laboratory, as was tritium-labeled poly A, specific activity 16 Ci/mM polynucleotide phosphorous (Lot 57-23-303), and tritium-labeled poly U, specific activity 36 Ci/mM polynucleotide phosphorous, (Lot 57-33-310). Deoxyribonuclease (DNase) and ribonuclease (RNase) were obtained from Worthington Biochemical Corp., Freehold, N. J. Digestion of nucleic acids with nucleases was carried out for 60 min at 37°C . (DNase 25 $\mu\text{g}/\text{mg}$ DNA; RNase 9 $\mu\text{g}/\text{mg}$ RNA). Pronase was obtained from California Biochemical Corp., Los Angeles, Calif. It was self digested for 4 hr at 37°C , followed by dialysis for 18 hr against continually changing deionized water. Proteolytic activity was verified by solubilization of ethanol-precipitable bovine gamma globulin (BGG). Uridine 5-T- ^3H , specific activity 8–15 Ci/mM, was obtained from Nuclear Chicago, Chicago, Ill. Liquoflor was purchased from Pilot Chemical Company, Long Island City, N. Y. for use as the scintillator in the standard counting solution, Ditol. Actinomycin D (Hoffman-LaRoche, Inc., Nutley, N. J.) was dissolved in 50% acetone and stored at -20°C .

PEC Isolation.—Macrophages (used synonymously with PEC) were isolated by a modification of the method of Gallily and Feldman (4). BALB/aj mice, 8 wk old, inbred in this department, were injected with 3.0 ml of thioglycollate medium. 4 days later the cells in the peritoneal cavity were washed out with 8.0 ml of phosphate-buffered saline (PBS), pH 7.2, (NaCl, 4.27 g/liter, Na_2HPO_4 , 8.10 g/liter; KH_2PO_4 , 2.45 g/liter) containing penicillin, streptomycin, and heparin (100 units, 50 μg , and 5 units USP/ml respectively). The cells were centrifuged at 600 rpm for 10 min and resuspended in 10 ml of Eagle's basal medium (BME) containing penicillin, 100 units/ml, and streptomycin, 50 $\mu\text{g}/\text{ml}$. Purification was achieved by centrifugation at 300 rpm for 10 min, removal by aspiration of the supernatant fluid and top 1–2 mm packed cells, resuspension of the pellet in 10 ml PBS, pH 7.2, and repetition of the centrifugation 2 times. Finally, the pellet was centrifuged at 900 rpm for 10 min, the supernatant fluid discarded, and five parts distilled water added to the packed cells with mixing to lyse red cells. The cells were mixed for 20 sec, immediately diluted 10-fold with BME, and counted in a hemocytometer or after staining with Wright's stain. The cell population was routinely greater than 95% macrophages and contained approximately 1% lymphocytes.

Preparation of Spleen Cells from Mice.—Spleens were removed from 20 mice, washed with PBS, pH 7.2, and extraneous fascia and connective tissue were trimmed. Four spleens at one time were minced in 10 ml PBS, pH 7.2, placed in a glass homogenizer (Potter-Evehjem), and homogenized 20 strokes. The homogenate was passed through a fine mesh screen, U.S. Bureau of Standards No. 80, and the cells were collected by centrifugation at 1500 rpm for 10 min. After washing 3 times with PBS, pH 7.2, the red cells were lysed with a 20 sec distilled water shock (3 v/v packed cells). Eagle's basal medium, minus serum, was added,

and the cells were centrifuged at 1500 rpm for 10 min. The pellet was resuspended in 40 ml BME with 20% calf serum containing penicillin and streptomycin. Adherent cells were separated from the spleen cell preparation by culture for 2.0 hr on Falcon plastic "T" flasks (Falcon Plastics, Los Angeles, Calif.) with a useable area of 30 cm.² The nonadherent cells were decanted, washed twice in BME minus serum, and added to monolayers of macrophages at 4.3×10^7 spleen cells/ml.

In Vitro Incubation of Macrophages with Antigen, Uridine, and Poly A:U.—For uridine labeling, 4 ml of peritoneal macrophages ($1-2 \times 10^7$ /ml), purified by centrifugation as described above, were placed in stoppered, siliconized Erlenmeyer flasks under an atmosphere of 95:5% air:CO₂ in BME without serum, but containing penicillin and streptomycin. The cells were incubated with BGG and poly A:U for 30 min at 37°C with shaking before the addition of uridine, 5-T-³H. 0.01 mCi contained in 0.002 μ mole of uridine was added to each flask and incubation carried out for an additional time period. Portions were removed into frozen, slushed PBS, pH 7.2, to stop uptake. The cells then were washed three times in PBS, pH 7.2, and finally precipitated with 2 N-HClO₄ containing 1% Dicalite in ice. After 30 min in ice the precipitate was centrifuged at 1000 rpm for 10 min and the pellet washed an additional three times with 5 ml 0.5 N-HClO₄. 0.2 ml of 0.5 N-HClO₄ was added to the pellet and each was boiled for 5 min. The whole pellet was then transferred to counting vials with 12 ml of Diotol scintillation fluid and counted in a model 3300 Packard scintillation counter (Packard Instrument Co., Downer's Grove, Ill.). Control, nonspecific absorption values were measured also by adding equivalent amounts of protein to the label after the addition of acid. Results were expressed as net cpm after subtracting background and control values. Replicate counts on separate samples were always within $\pm 5\%$.

For reinjection into mice, peritoneal macrophages were obtained and purified as described above. Incubation with antigen was carried out for 2 hr after which the cells were washed four times with 50 volumes/cell pack of cold BME at 1000 rpm for 20 min. The cells were resuspended at $3-5 \times 10^7$ /ml and 0.5 ml injected intraperitoneally into normal 7-8-wk old mice. Control mice also were injected with BGG and poly A:U in 0.5 ml without macrophages. Blood was collected on various days and the serum assayed by passive hemagglutination using sheep red cells sensitized with 3 mg BGG/ml 2.5% cells.

Isolation of RNA from Macrophages.—Nucleic acids were prepared essentially free of protein and containing no nucleotides or nucleosides by the method of Marmur (13) as modified for RNA by Adler et al. (14). A 7.0 ml packed cell volume of purified macrophages, which had been stored at -20°C , was thawed in one volume of buffer solution, pH 5.0, containing 10^{-3}M MgCl₂, 0.2 M LiCl, 0.01 M acetic acid, and 0.01 M sodium acetate and 0.5% SDS. The mixture was shaken vigorously for 3 min after the addition of one volume of freshly distilled, water-saturated phenol. It was then heated at 60°C for 3 min, cooled rapidly, and centrifuged at 12,000 rpm for 30 min. The pellet was reextracted once with phenol and the aqueous phases combined. The aqueous phase was layered with six volumes of cold absolute ethanol, allowed to stand for 24 hr at -20°C , and centrifuged at 17,000 rpm for 30 min. The pellet was resuspended in 5 ml of buffer (0.02 M tris(hydroxymethyl)aminomethane (Tris), pH 7.6, 10^{-3}M MgCl₂, 0.2 M LiCl). The ethanol precipitation was repeated and the final pellet resuspended in the above buffer without LiCl. 20 mg RNA was digested with 100 μg /ml DNase I for 1 hr at 37°C. After DNase treatment the RNA was precipitated by four volumes of absolute ethanol and centrifuged at 17,000 rpm for 60 min. The pellet was resuspended in the above buffer minus LiCl. This solution was then dialyzed overnight against 500 ml of buffer.

RNA Prepared for Sucrose Density Gradients and Biological Activity.—To obtain RNA for determination of a species of mRNA which could be quickly labeled by uridine, a modification of the method of Ralph and Bellamy (15) was used. Macrophages were induced with thioglycolate and harvested. After purification and incubation with antigen, uridine, and poly

A:U, the macrophages were washed four times with BME and resuspended in two volumes of buffer (0.15 M NaCl, 0.1 M ethylenediamine tetraacetate (EDTA) containing 10^{-3} M $MgCl_2$ plus 0.5% SDS). The cells were then homogenized for 20 strokes in a glass homogenizer, an equal volume of freshly distilled phenol was added, and the mixture was shaken for 10 min at 25°C and stirred for 3 min at 63°C. The aqueous phase was removed after centrifugation at 1000 rpm for 15 min. An equal vol of chloroform-isoamyl alcohol was added to deproteinize the material, and after shaking for 10 min the mixture was centrifuged at 1000 rpm for 10 min. This deproteinization was repeated three times. The aqueous phase was divided, and to one half was added four volumes of cold, absolute ethanol, and precipitation was allowed for 24 hr at -20°C. The other half was treated for 60 min with 1 mg self-digested Pronase/ml of nucleic acid. The Pronase treated material was deproteinized as above, and precipitated with absolute ethanol. Both precipitates were resuspended in small volumes of 0.2 M LiCl, 0.02 M Tris, pH 7.8, 10^{-3} M $MgCl_2$, and concentrations determined by absorption at 260 $m\mu$.

Characterization of RNA by Density Gradient Centrifugation.—Molecular sizing of the newly synthesized RNA under the stimulation of antigen was accomplished by the use of sucrose buoyant density centrifugation methods. Linear sucrose gradients were poured in SW 40 Spinco tubes (Beckman Instruments, Inc., Fullerton, Calif.) 95 mm, with a total volume of 12.5 ml containing 5–20% sucrose, 5×10^{-3} M $MgCl_2$, 0.05 M Tris, pH 7.8, and 0.5% SDS. 0.5 ml of RNA solution containing 1–3 mg RNA was layered on the top and the gradients were spun at 39,000 rpm for 150 min at 20°C to keep the SDS in solution. After centrifugation the gradients were removed and 25–30 samples collected by catching drops from holes punched in the gradient. The location of various sized molecules was determined by analyzing the absorbancy of each fraction at 260 $m\mu$ and counting the total fraction in 12 ml of Diotol solution in a Packard 3300 scintillation counter.

Trace Iodination of BGG.—The chloramine-T method of McConahey and Dixon (16) was used and specific activities of 2–40 mCi/mg protein were obtained.

Passive Rosette Assay.—The red cell rosette test (17, 18), as adapted for protein antigens by R. D. Stout (Ph.D. thesis, 1970, The University of Michigan), was used to characterize the kinetics of antibody-forming cell populations in the spleen during the immune response of mice to BGG or macrophage RNA. Sheep red blood cells (SRBC) were washed three times in PBS, pH 7.2, and resuspended to a 50% concentration. 0.5 mg (100 mg/ml) ECDI (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl) obtained from the Ott Chemical Co. Muskegon, Mich., was added to 3 ml BGG (15 mg/ml) and 0.1 ml 50% SRBC. The mixture was incubated for 60 min at 4°C, then centrifuged at 1500 rpm for 15 min. The red cells were washed twice in 1.0% normal rabbit serum in PBS, pH 7.2. Finally the cells were re-suspended at a concentration of 7.0% (19). Accuracy of the rosette assay was maintained when the number of rosettes counted varied between 20 and 200. The concentration of spleen cells used was $1-10 \times 10^6$ /ml. 0.1 ml of 7.0% sensitized SRBC cells was added to a 51 \times 60 mm tube containing the test spleen cells. The tubes were incubated at a 45° angle for 2 hr and overnight at 4°C. The rosettes were counted in a hemocytometer and the final spleen cell count was determined by lysing SRBC in 2% acetic acid. The results are expressed as the number of rosette-forming cells (RFC) per 10^6 spleen cells.

RESULTS

The Adjuvant Action after Exposure of Antigen to Macrophages and its Enhancement by Homoribopolymer Complexes.—When macrophages isolated from the peritoneal exudate of thioglycolate-treated, normal mice were exposed in vitro to BGG and reinjected into syngeneic mice, a shortened induction period and increased antibody titers were elicited, as compared to mice injected di-

rectly with antigen. Representative data are shown in Table I. Addition of poly A:U to the incubation mixture further increased these titers. Additional experiments established that both 2-mercaptoethanol-sensitive and insensitive antibody titers were elevated by exposure of antigen to macrophages and poly A:U. The adjuvant action of the double helix made up of the homoribopolymers, polyinosinic and polycytidylic acids (poly I:C), also was tested in this system, and these complexes exerted a similar enhancing capacity over and above that produced by antigen alone when added to macrophages.

The relationship between decreasing numbers of macrophages incubated with antigen and the magnitude of the stimulation of antibody synthesis is seen in Table II. A drastic reduction in the effect was observed when the cell

TABLE I
The Adjuvant Action of Antigen Exposure to Macrophages and its Further Enhancement by Poly A:U

Mixture injected*	Antibody Titer		
	Days after injection		
	6	8	10
Macrophages only	0	0	0
BGG only	80	320	640
BGG + Poly A:U	160	2,560	10,240
Macrophages + BGG	320	5,120	10,240
Macrophages + BGG + Poly A:U	640	10,240	40,960
Macrophages + Poly A:U	0	0	0

* Macrophages, 2.4×10^7 per mouse; BGG, 0.5 mg; and Poly A:U, 150 μ g of each polymer, were incubated together (or separately as controls) for 2 hr with shaking, washed 4 times, and reinjected intraperitoneally into syngeneic mice. Antibody levels are the reciprocal of the passive hemagglutination titer, determined 6, 8, and 10 days after injection of recipient mice.

population was decreased from 10^7 to 10^6 /ml, a fact also recorded by others (4, 20). A minimal number of 10^7 macrophages, necessary for eliciting increased antibody synthesis by macrophages and antigen without adjuvant, was also necessary for the further adjuvant action of homoribopolymers. This suggested that poly A:U was increasing a process required to be initiated by an adequate number of macrophages and was not merely additive to the macrophage effect. The injection of 10^5 cells/ml suppressed the response and poly A:U was unable to stimulate 10^5 macrophages exposed to 0.5 mg of BGG to evoke an enhanced response in mice. This was despite the fact that poly A:U, when injected in vivo with antigen, was capable of stimulating an antibody response to as little as 1 ng of BGG.² Thus, a level of transfer or concentration of antigen below 10^{-9} g BGG by 10^5 macrophages was indicated. It had been shown previously² that a single injection of poly A:U 1 day before antigen

suppressed rather than enhanced antibody synthesis. To test whether this immunosuppression might be due to a defect in macrophage handling of antigen *in vivo* induced by prior injection of poly A:U, macrophages were removed from mice injected with poly A:U 18 hr before harvesting (2 days after thioglycolate).

TABLE II
Titration of Numbers of Macrophages Required for Adjuvant Action

Mixture injected*	Antibody Titer	
	Days after injection	
	7	10
BGG only	1,280	5,120
BGG + Poly A:U	2,560	10,240
Macrophages, 5.0×10^7 + BGG	10,240	10,240
Macrophages, 5.0×10^7 + BGG + Poly A:U	20,480	40,960
Macrophages, 5.0×10^6 + BGG	80	80
Macrophages, 5.0×10^6 + BGG + Poly A:U	40	40
Macrophages, 5.0×10^5 + BGG	0	0
Macrophages, 5.0×10^5 + BGG + Poly A:U	0	0

* BGG, 0.5 mg; Poly A:U, 150 μ g each. Incubation as in Table I.

TABLE III
Loss of Adjuvant Action of Macrophages after Pretreatment of Mice with Homoribopolymers

Mixture injected*	Hemagglutination titer		
	Days		
	6	8	10
BGG	40	160	320
BGG + Poly A:U	80	640	640
Macrophages + BGG	160	1280	2560
Macrophages + BGG + Poly A:U	640	5120	20480
Macrophages \ddagger + BGG	40	320	320
Macrophages \ddagger + BGG + Poly A:U	80	160	320

* BGG, 0.5 mg; poly A:U, 150 μ g/ml each; macrophages, 4.4×10^7 /ml. Incubation as in Table I.

\ddagger Macrophages obtained from mice injected with 300 μ g poly A:U 18 hr before harvesting.

The results (Table III) supported this hypothesis in that these PEC were found to have lost the capacity to enhance antibody synthesis.

The act of inducing a sterile peritonitis by injecting thioglycolate medium into the mouse might give rise to PEC with abnormal properties. Such cells could have been stimulated to a high degree by the presence of the irritant, or by endotoxin contamination, resulting in the adjuvant action. However, in

repeated experiments the injection of 4 ml of sterile pyrogen-free PBS, pH 7.2, resulted in PEC which also were capable of similar enhancement of antibody synthesis, despite being smaller and 100-fold less in number per mouse than those induced with thioglycolate. It was concluded that saline-induced PEC do not differ in adjuvant properties from thioglycolate-induced cells. A similar conclusion was reached by Mitchison (8).

Evidence has accumulated recently that the interaction of two or more cells may be required for antibody synthesis in response to some antigens (2, 3). To

TABLE IV
Effect of Interaction of Competent Macrophages with Spleen Nonadhering Cells

Incubation mixture injected	HA antibody titer		
	Days after inoculation		
	6	8	10
BGG	80	320	160
Reisolated spleen cells after interaction with competent macrophages	2560	1280	1280
Macrophages incubated with BGG without spleen cells	1280	1280	320

Concentrations: BGG (agg), 0.5 mg; macrophages, 5×10^7 /ml; spleen cells, 4.3×10^7 /ml.

TABLE V
The Effect of BGG and Poly A:U on the Uptake of ^3H -Uridine into Macrophages

Mixture*	Net CPM into RNA	
	15 min	60 min
Macrophages	8796	16123
Macrophages + BGG	12374	19202
Macrophages + Poly A:U	10766	18022
Macrophages + BGG + Poly A:U	15552	23472

* Macrophages, 2×10^7 ; BGG, 0.5 mg; Poly A:U, 150 μg each.

determine if macrophages exposed to antigen could transfer their adjuvant capabilities to another cell, the following experiment was performed. Monolayers of macrophages were prepared, aggregated BGG was added, and the mixture was incubated for 30 min. The macrophages were washed four times and purified spleen, non-glass-adhering cells were added over the macrophage monolayer. The cells were allowed to interact for 2 hr after which the nonadhering spleen cells, which were 90% lymphocytes, were decanted, washed four times, resuspended, and injected into normal mice. Table IV shows that the lymphocyte-rich population indeed had acquired the capacity of provoking an enhanced antibody response as compared to antigen injected alone. Inas-

much as the data in Table II indicated that less than 10^7 macrophages were incapable of enhancing antibody synthesis, it is unlikely that the effect was due to contamination of the lymphocytes with macrophages. Macrophages exposed to antigen which did not have any spleen cells added to them remained capable of adjuvant action, as expected. However, in control tests, the medium alone in which only macrophages had been incubated was not able to induce circulating antibody, indicating there had been no detectable release of immunogenic factors into the medium. The effect of poly A:U on these experiments is under study.

Nature of the Material Stimulated in Macrophages.—If *de novo* synthesis of RNA were a molecular event in cells that followed recognition of nonself molecules, an increase in uridine uptake into RNA should have been apparent

TABLE VI
The Effect of Actinomycin D on the Adjuvant Action of Peritoneal Macrophages with BGG and Poly A:U

Incubation mixture injected*	HA antibody titer		
	7 days	9 days	11 days
Macrophages only	0	0	0
BGG only	10	20	10
BGG + Poly A:U	320	640	1280
Macrophages + BGG	160	320	320
Macrophages + BGG + Poly A:U	640	1280	5120
Macrophages + BGG + ActD	20	10	20
Macrophages + BGG + Poly A:U + Act D	80	160	640

* Concentrations: macrophages, 2.4×10^7 per mouse; BGG, 0.5 mg; poly A:U, 150 μ g each; actinomycin D (Act D), 15 μ g.

when macrophages were incubated in the presence of antigen. This correlation did exist, as is shown in Table V. When BGG and poly A:U were added in combination, a doubling of the uptake of label occurred. In addition, poly A:U without antigen was capable of causing a stimulation in the uptake of label into RNA. The optimum concentration of BGG effective at increasing uridine labeling was found to be 0.5–1 mg/ml, while 0.25 mg/ml had little effect. Inhibition was seen with 2 mg/ml. The optimum concentration of poly A:U resulting in maximum uptake appeared to be 150–300 μ g/ml; little stimulation occurred at 75 μ g/ml. No further increase in stimulation could be seen with poly A:U concentrations up to 2400 μ g/ml. Relative values similar to those shown in Table V were obtained repeatedly.

To determine any possible relationship of this newly synthesized RNA to messenger RNA, actinomycin D was added to the macrophages in culture before the addition of antigen or poly A:U. After incubation with the latter, the

macrophages were washed four times to insure that the level of carry-over of residual actinomycin D into the mouse was far below the value of 10 μg of actinomycin D per mouse, shown by Wust et al. (21) to have no effect on subsequent antibody synthesis. It can be seen in Table VI that 15 μg of actinomycin D effectively blocked the ability of the macrophage to enhance antibody synthesis, thus suggesting that the role of the macrophage includes synthesis of messenger RNA. Of interest is the fact that actinomycin D inhibition was reversed by poly A:U when the latter was given either directly before or after this inhibitor. Jaroslow (22), using RNA-RNase digests, showed a similar inhibition by actinomycin D of antibody synthesis in rat lymph node cultures.

Study of the effect of the time of addition of actinomycin D during the in

TABLE VII
Effect of Time of Addition of Actinomycin D to Macrophage-Antigen Mixtures

Incubation mixture injected	Act D added	HA antibody titer		
		Days after inoculation		
		6	8	10
	<i>min.</i>			
BGG	—	160	640	320
Macrophages + BGG	—	640	2560	2560
Macrophages + BGG	0	0	0	0
Macrophages + BGG	15	40	80	160
Macrophages + BGG	30	20	160	320
Macrophages + BGG	60	20	160	320
Macrophages + BGG	120	640	640	320
Macrophages + BGG 0.5 mg added at time of injection	0	320	320	640

Concentrations: BGG (agg), 0.5 mg; macrophages, 2.5×10^7 /ml; Actinomycin D (Act D), 15 μg / ml.

vitro incubation of macrophages with antigen confirmed that complete inhibition occurred if actinomycin D was added at zero time; however, if actinomycin D was delayed for only 15 min after antigen had been added, some RNA was synthesized, as denoted by measurable antibody titers (Table VII). If the drug were added at 120 min, antibody titers were comparable to those seen with antigen injected without macrophages. The data shown in the last line of Table VII represents the control mixture. In this sample actinomycin D was added at zero time and the mixture was incubated without antigen for 120 min. After washing the macrophages and resuspending them in PBS, pH 7.2, antigen was added immediately before injection. If inhibitory concentrations of actinomycin D were carried over to the recipient mouse, antibody synthesis should have been inhibited in these mice. Since it was not, it was concluded that the adjuvant action of macrophages required metabolically active cells and depended upon messenger RNA synthesis. However, there was no evidence from

this experiment that the required messenger RNA was coded for immunoglobulin synthesis.

The nature of the newly synthesized macrophage RNA was examined further by comparing the distribution of macrophage RNA from macrophages exposed or not exposed to antigen after sucrose buoyant-density centrifugation. A marker (poly A) with a known S value of eight was included in the gradient so that relative S values could be assigned to the RNA synthesized under

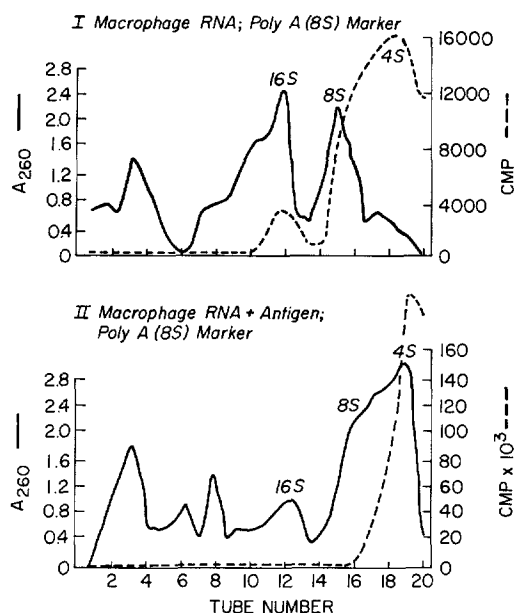


FIG. 1. The distribution of macrophage RNA after antigenic stimulation *in vitro* by BGG. The RNA was isolated by hot phenol. Gradients were composed of 5–20% sucrose containing $5 \times 10^{-3}M$ $MgCl_2$; 0.05 M Tris, pH 7.8; 0.5% SDS, and was centrifuged at 39,000 rpm for 150 min. The tubes were divided into 20 fractions and each was analyzed for radioactivity and A_{260} .

antigenic stimulation. From Fig. 1 it may be seen that there was a shift of 16S ribosomal RNA to the smaller 4S RNA when macrophages were exposed to antigen. The latter RNA, however, would be of insufficient size to be messenger RNA.

The immunologic competency of this newly synthesized RNA was tested following its isolation by phenol. The RNA-rich material (termed RNA) isolated under these conditions proved occasionally to be toxic for mice, and only small amounts were tolerated. All attempts to effect an antibody response by injecting RNA alone met with failure, except, paradoxically, those instances in

which RNA was treated with the self-digesting enzyme, Pronase, in an attempt to eliminate any response by removing antigenic fragments postulated as being attached to the nucleic acid. On the other hand, when poly A:U was added to the RNA at the time of injection, antibody titers detectable by passive hemagglutination were elicited. Table VIII shows results representative of six different positive experiments. In instances where ^{131}I -labeled BGG was used as antigen, antigenic retention measured isotopically was found to be 0.1 ng/ml or less after the isolated RNA was treated with Pronase. Consequently, by this technique the greatest amount of antigen given with RNA to any given mouse appeared to be on the order of 10^{-11} g. Yet this RNA was immunogenic, capable of eliciting a slight, but reproducible and specific antibody response under the

TABLE VIII
Induction of Rosette-Forming Cells and Hemagglutination Antibody after Injection with Macrophage RNA

Condition	No. of rosettes per 10^6 spleen cells Day 4	HA antibody titer			
		Day			
		6	32	35	38
BGG	7,800	40	10	0	10,240
RNA 0.070 mg	6,500	0	0	0	2
RNA 0.070 mg + Poly A:U	10,000	8	0	0	16
P-RNA 0.063 mg	5,500	4	0	0	32
P-RNA 0.063 mg + Poly A:U	9,600	16	0	0	64
P-RNA 0.063 mg + RNase	1,200	0	0	0	20
Control	1,400	0	—	—	—

Concentrations: BGG, 0.50 mg; poly A:U, 150 $\mu\text{g}/\text{ml}$ each; RNase, 25 $\mu\text{g}/\text{mg}$ RNA; incubated 60 min at 37°C. All mice, except control group, were reinjected with 0.50 mg BGG on day 32. P-RNA represents RNA treated with Pronase.

stimulation of poly A:U. The effectiveness of this immunogenicity is to be compared with 10^{-6} g of antigen found as required to be retained in preliminary experiments for detectable antibody synthesis after exposure to viable whole macrophages and poly A:U.

Since the titers of antibody induced by macrophage RNA were low, the numbers of rosette-forming cells in the spleen were also measured after RNA injection. The RFC appearing on day 4 are compared in Table VIII with the antibody titers appearing 2 days later in other groups of similarly injected mice. It may be seen that the RNA induced RFC comparable in number to those stimulated by injection of BGG alone. Again, poly A:U given with RNA elevated the RFC in comparison to RNA alone. It also may be seen that RNase digestion of the RNA preparation completely reduced the RFC to control levels. In addition, the data in Table VIII record that the mice induced by RNA

were incapable of responding to a second injection of antigen with a typical secondary antibody response. All RNA-injected mice showed low levels of circulating antibody on day 38, 6 days after reinjection of BGG, in contrast to the group initially injected with antigen rather than RNA, which showed a titer of 10,240. Because none of the animals primed with RNA even approached this level on reinjection of antigen, any antigenic fragment, if present in the RNA, differed either quantitatively or qualitatively from the original antigen in capacity to prime for a secondary response. Table IX records one of the confirmatory experiments in which the background count of RFC was down to lower levels, 495 RFC/10⁶ spleen cells. In this experiment again, the RNA-rich preparation which had been digested with Pronase was able to induce RFC comparable in number to the RFC induced by injected antigen alone.

TABLE IX
Induction of Antibody Synthesis by an RNA-Rich Fraction Isolated from Macrophages

Material injected	RFC/10 ⁶ spleen cells	HA antibody titer	
		Day 6	Day 35
BGG	6,400	40	20,480
RNA, 0.1 mg	2,000	0	40
RNA, 0.1 mg + poly A:U	5,250	2	40
RNA,* 0.2 mg	5,300	4	20
RNA,* 0.2 mg + poly A:U	8,700	16	320
Control	495	0	ND

Concentrations: BGG, 0.5 mg; poly A:U, 150 μ g each/ml. RFC measured on day 6 after injection. All mice injected with BGG 30 days after initial injection of BGG or RNA.

ND = not done.

*RNA treated with Pronase.

Poly A:U was able to elicit greater numbers of RFC/10⁶ spleen cells than RNA without the adjuvant, and once again, the secondary response in these RNA-induced mice was much less than that seen in mice stimulated with antigen alone.

DISCUSSION

The macrophage limb of the immune system and its role as a possible locus for the enhancing action of poly A:U was investigated in these experiments. A possible function for the macrophage in antibody synthesis became apparent from the experimental model developed by Fishman (1). With this model, anti-T2 phage antibody was elicited by incubating an RNA-rich preparation from rat peritoneal exudate cells exposed to antigen with lymph node cells. Although in confirmatory studies by others (23-25), it was shown that similar, unfractionated RNA preparations contained antigen capable of reacting with

anti-phage antibody; in further experiments (10), three fractions with approximate S values of 4-5, 16, and 23-28 could be separated on sucrose density gradients from the RNA preparation, and antigen was detected only in the heavy RNA. The antibody response was biphasic with a peak of 19S antibody elicited by the light RNA at 2 days and a peak of 7S antibody evoked by the heavier RNA at 11 days. Thus, it was suggested that only the activity of the heavy RNA fraction was dependent on the presence of antigen or antigen fragments.

The chemical nature of the RNA-antigen complex has been explored by Roelants and Goodman (26) and by Gottlieb (27), with disagreement as to its general biological significance. However, the immunogenic activity of antigen bound to RNA was much greater than that of free antigen irrespective of how it was achieved. The existence of RNA-antigen complexes in mouse liver had been established earlier by Garvey and Campbell (28), and these complexes likewise proved to be more efficient than the original antigen. Thus, the potentiality of poor immunogens (e.g. viruses and tumor antigens) made much more efficacious by combination *in vitro* with cellular RNA, is an important offshoot of these and our studies, even though an active processing or concentrating role of the macrophage in antibody formation *in vivo* has not yet been proven.

The possibility that the light RNA was an informational macromolecule was raised in the experiments of Adler et al. (14) who incubated RNA extracted from peritoneal cells of rabbits of one allotype with lymph node fragments removed from rabbits of a different allotype. The resulting antibody produced by the lymph node fragments proved to be of the same allotype as the donor of the peritoneal cells. However, in our study, as well as several others (10, 26, 29) the active RNA extracted from macrophages has been estimated to be too small to direct the synthesis of either a light or heavy immunoglobulin chain.

Using the findings described above as a guide, the phases at which the polynucleotide adjuvants might be operative were tested. Our data show that antigenic stimulation of macrophages caused an increase in uridine uptake which could be furthered by poly A:U. Each increased RNA synthesis correlated with elevated antibody levels. Actinomycin D established that viability and synthesis of messenger RNA were necessities for the adjuvant action of this adherent cell. This confirmed the finding of Bishop et al. (29), but is to be distinguished from the chemical study of Gottlieb (27), in which actinomycin D failed to impair formation of the RNA-antigen complex. However, mere incubation of RNA from normal macrophages not exposed to antigen with small amounts of free antigen did not result in a complex being formed.

From the data in Table VII, it is evident that less than 15 min exposure to antigen and poly A:U was required for whatever macromolecular synthesis was necessary for enhancement. However, our sucrose buoyant-density centrifugation experiments showed the biologically active, transferrable RNA to be

primarily of the 4S size, indicating the required messenger RNA was not synonymous with this entity.

Surprisingly, the 4S RNA after injection in mice was able to induce a measurable, specific antibody titer, as well as rosette-forming cells, if treated with Pronase and stimulated by poly A:U. It is of interest that Fishman and Adler (10) reported abolition of late antibody synthesis by the heavy RNA, but not the early 19S antibody synthesized by the 5S RNA, when both were treated with Pronase. Contamination of our RNA with isotopically labeled antigen was below the level of 10^{-11} g, but further data are necessary to support the possibility that antibody formation was not initiated by antigenic fragments. Data supportive of the hypothesis that macrophages can synthesize information RNA after stimulation by antigen has been recorded in a series of important studies by Jacherts (30). In these experiments, 19S anti-phage antibody synthesis was initiated both in spleen cell cultures and in a cell-free system by several sizes of RNA.

SUMMARY

Incubation of antigen with normal mouse peritoneal exudate cells in vitro and subsequent reinjection of the washed cells into syngeneic mice resulted in increased antibody titers as compared to mice injected with antigen alone. Several of the variables influencing this system were studied with and without the stimulus of complex homoribopolynucleotides (poly A:U or poly I:C) as adjuvants to determine the cellular site of action of the latter. It was found that addition of poly A:U or poly I:C caused a further rise in circulating antibody levels which correlated with increased RNA synthesis, suggesting that the macrophage was one cell affected by this adjuvant. Actinomycin D was found to inhibit the rise in titer induced by PEC and this inhibition could be overcome by poly A:U. Injection of the polynucleotides 18 hr before antigen resulted in depression of circulating antibody levels, and poly A:U or poly I:C injected 18 hr before harvesting PEC and incubation with antigen also inhibited the capacity of the PEC to increase antibody levels.

A 4S RNA-rich fraction was purified after treatment with phenol of PEC exposed to antigen in vitro, and under the stimulus of poly A:U this RNA was capable of inducing specific antibody titers and rosette-forming cells on injection into mice. Antigen contamination of Pronase-treated RNA, active biologically, was below 10^{-11} g as determined isotopically.

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