THE CHEMICAL NATURE OF MACROPHAGE RNA-ANTIGEN COMPLEXES AND THEIR RELEVANCE TO IMMUNE INDUCTION*

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An important role for RNA complexed with antigen or fragments of antigen in the induction of the immune response has been postulated (1-4).

The source of these complexes is believed to be the macrophage, which takes up and processes foreign matter. While the precise role of the macrophage in antibody induction has not yet been elucidated (5), it has been hypothesized that after exposure to antigen the macrophage transmits an informational RNA to lymphoid cells (6). This concept was based on the finding that RNA extracted from peritoneal exudate (PE) cells after exposure to antigen induced the synthesis of specific antibody by unprimed lymphoid cells. Subsequently, the presence of antigen was demonstrated in similar RNA preparations, raising questions about the role of RNA in the experimental system (7, 8).

A previous report from this laboratory (9) compared the association with rabbit PE cell RNA of two forms of radiolabeled poly- γ -D-glutamic acid, the free polypeptide which appears to be nonimmunogenic by several criteria (10, 11), and the polypeptide complexed with methylated albumin, which regularly elicits antibody reactive with the polypeptide itself. The central findings of that study were that very stable complexes were formed between a fraction of the polypeptide taken up by the cells and 4-5S RNA. The labeled polypeptide could not be dissociated from RNA by sucrose density gradient centrifugation, adsorption chromatography, gel electrophoresis, equilibrium sedimentation in cesium sulfate, or in competition by 10^5 -fold excesses of unlabeled polypeptide. Furthermore, there was no association between purified RNA and the polypeptide mixed in vitro, but association comparable to that obtained with intact cells occurred with cell-free homogenates. These results prompted the conclusion that the association of poly- γ -D-glutamic acid with macrophage RNA is an active biological process and that the association is very strong and possibly covalent. However, there was no discernible qualitative or quantitative distinction

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between the two forms of the polypeptide, so that association with RNA did not appear to distinguish between molecules on the basis of immunogenicity.

In order to assess the role of antigen-RNA complexes in immune induction, these studies were extended. The present report is addressed to the following questions.

1. What is the relationship between molecular structure and immunopotency of the "antigen" and its capacity to form complexes with RNA?
2. Is complex formation an enzyme-dependent reaction?
3. Does it involve "antigen-specific" RNAs?
4. Does it require newly synthesized RNA?
5. Can it occur only with macrophage RNA?
6. What is the chemical nature of the RNA-antigen bond?

Materials and Methods

"Antigens."—*Poly- γ -D-glutamic acid*: The purification and characterization of the capsular polypeptide of *Bacillus anthracis* strain M36 (12), its electrostatic complexing with methylated bovine serum albumin (MBSA) (10), and the purification and characterization of the polypeptide radiolabeled by exposure to tritium gas (9) have been described. The polypeptide, of average molecular weight 33,500 (12), had a specific radioactivity of 51 μ c/mg after tritiation. The labeling procedure did not produce significant racemization, as ascertained by gas-liquid chromatography (9).

Synthetic poly- α -D-glutamic acid (Yeda, Israel) of average molecular weight 42,000 was radiolabeled, purified, and characterized by the same procedures used for poly- γ -D-glutamic acid. The specific radioactivity of the labeled product was 147 μ c/mg.

14 COOH-dextran (New England Nuclear Corp. Boston, Mass.) of a molecular weight ranging from 60,000 to 90,000 had a specific radioactivity of 0.87 mc/g. The labeling was achieved by carboxylation of the original dextran molecule.

Dextran from *Leuconostoc mesenteroides* NRRL B-1399, fraction S, from the Agricultural Research Service, U. S. Department of Agriculture, was radiolabeled by exposure to tritium gas (New England Nuclear Corp.). After purification it had a specific radioactivity of 96 μ c/mg and differed from 14 COOH-dextran in that it lacked carboxyl groups.

A sample of the multichain synthetic polypeptide poly-(tyr, Glu)-poly-DL-Ala-poly-Lys [(TGAL)], labeled with 125 I (2.18 μ c/ μ g), was provided by Dr. H. McDevitt.

A human γ G myeloma protein, labeled with 125 I (40.5 μ c/mg), was provided by Dr. H. Fudenberg.

Several radioactive products were obtained from New England Nuclear Corp., including 14 C-testosterone (50.6 mc/mmmole), 3 H-cortisone (34.4 c/mmmole), 3 H-estradiol (40.0 c/mmmole), 14 C-L-glutamic acid (218 mc/mmmole), and 14 C-uridine (0.211 mc/mg); 14 C-DL-glutamic acid (6.7 mc/mmmole) was obtained from Calbiochem, Los Angeles, Calif.

Animals.—The animals used in these experiments were randomly bred rabbits and guinea pigs. For experiments involving (TGAL), mice of strains CBA and C57, generously donated by Dr. H. McDevitt, were employed.

Exposure of Peritoneal Exudate Cells to "Antigens" and Subsequent Purification of RNA.—The harvesting and culture of PE cells obtained following intraperitoneal injection of sterile light mineral oil; the preparation of cell-free homogenates by disrupting the cells with a Dounce tissue grinder after suspension in hypotonic medium; the RNA preparation, which involved four cold phenol extractions and repeated precipitation by ethanol; and the characterization

of RNA by sucrose density gradient ultracentrifugation have been described (9). The purified preparations had a $260\text{ m}\mu/280\text{ m}\mu$ ratio of 2.0. The only modification adopted in the present investigation was that in standard experiments the cell homogenates were incubated with "antigen" in a total volume of 1 or 2 ml at 37°C for 20 min instead of 1 or 2.5 hr as before.

Labeled RNA was obtained by adding $5\text{ }\mu\text{C}$ of ^{14}C -uridine to a suspension of 10^9 cells at the time of addition of "antigen." Under the conditions used, only 4-5S RNA was significantly labeled. To block transcription, $600\text{ }\mu\text{g}$ of actinomycin D (Calbiochem) was added to 10^8 cells 45 min before the addition of ^{14}C -uridine and "antigen".

In some experiments, cell homogenates were treated with Pronase (Calbiochem, grade B) at a concentration of 5 mg/ml at 37°C for 8 hr, or with 15 mg/ml of dithiothreitol (P-L Biochemicals, Inc., Milwaukee, Wis.) for 30 min.

The ^3H and ^{14}C radioactivity was counted in a model 337S Packard Tri-Carb scintillation spectrometer, while the ^{125}I was quantitated in a Packard Auto-Gamma spectrometer.

HeLa cells were kindly provided by Dr. Leon Levintow.

Subcellular Fractionation.—Intact peritoneal exudate cells, incubated with ^3H -poly- γ -D-glutamic acid and washed until significant radioactivity could no longer be detected in the washes, were fractionated according to Mathias (13). The cells were suspended in 5 ml of buffer containing 0.03 M Tris-HCl (pH 7.3), 0.003 M CaCl_2 , and 0.25 M sucrose and were disrupted with five strokes of a Teflon homogenizer. The homogenate was centrifuged at 700 g for 10 min, after which the supernatant was centrifuged at $15,000\text{ g}$ for 10 min. The pellet was suspended in 0.5 ml of the same buffer and designated fraction I (mitochondria-lysosomes). The supernatant volume was adjusted to 5 ml with the same buffer and centrifuged at $100,000\text{ g}$ for 90 min (Spinco L2 preparative ultracentrifuge, SW 65K rotor). The supernatant from this run was designated fraction IV (soluble fraction).

The pellet was suspended in 5 ml of buffer containing 0.05 M Tris-HCl (pH 7.8), 0.0001 M MgCl_2 , 0.05 M KCl, 0.5% sodium deoxycholate, and 0.25 M sucrose and was centrifuged for 90 min at $100,000\text{ g}$. The supernatant was designated fraction II (endoplasmic reticulum-lipoprotein), and the pellet was called fraction III (ribosomes). No attempt was made to purify these fractions further.

Samples of 100 or 200 μl were taken from each fraction and put in liquid scintillation vials (The Radiochemical Center, Amersham, England). After the addition of 2 drops of hydrogen peroxide, digestion was performed with 2 ml of NCS solubilizer (G. D. Searle & Co., Chicago, Ill.) for 12 hr (14). The radioactivity was counted in the scintillation spectrometer after the addition of 18 ml of 6 g/liter of 2,5-diphenyloxazole (PPO) in toluene. Before reading, the scintillation vials were left for at least 5 hr in darkness at 4°C . Disintegrations per minute were derived from calibrated external standards.

In Vitro Association of "Antigens" with RNA.—Association in vitro was tested by incubation of labeled compounds with purified PE cell RNA at 25°C or 60°C during 10 min in 0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA, and 0.01% sodium dodecyl sulfate, with various concentrations of Mg^{++} . The chilled preparations were then reprecipitated three times with 2 volumes of ethanol before assay for radioactivity and RNA content. Some samples were extracted with phenol prior to precipitation with ethanol, but the additional purification did not significantly change the results.

Dissociation of the complexes in vitro was attempted by suspending known PE cell RNA- ^3H -poly- γ -D-glutamic acid complexes obtained in vivo in 1 ml of 0.02 M Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate, and 0.028 M EDTA, and dialyzing during 3 days at 20°C or 80°C against 250 ml of the same buffer changed twice daily. The same procedure with buffer devoid of EDTA was run as a control. After 3 days the chilled contents of the dialysis bags were precipitated twice with 2 volumes of ethanol, and the precipitates and supernatants were assayed for radioactivity and RNA content. The polypeptide/RNA ratio was expressed as a percentage of the ratio of the starting material.

RESULTS

Association of Various Molecules with PE Cell RNA.—A variety of different radiolabeled molecules were assayed for their capacity to bind with RNA in suspensions of PE cells or homogenates of the cells. Table I gives the dose range of the "antigens" used and their levels, based on specific radioactivity, in purified RNA. The "antigen" which survives the purification procedure has been shown to be physically associated with 4-5S RNA (9). The capacity to

TABLE I
The Association of Various Molecules with RNA from Peritoneal Exudate Cells

Molecule	Total dose range		No. of experiments	Efficiency of binding*
	μg	μc		
^3H -Poly- γ -D-glutamic acid‡	40-550	(2.04-28.05)	44§	$1.7-10 \times 10^{-4}$
^3H -Poly- α -D-glutamic acid	110-550	16.17-80.85	4	$1.8-4.0 \times 10^{-4}$
^{14}C -COOH-dextran	572-11450	0.49-9.96	20	$1.3-4.9 \times 10^{-4}$
^3H -Dextran	260	24.96	2	Below detection
^{125}I -Myeloma protein	40	1.62	2	4.7×10^{-5}
^{125}I -(TGAL)	4	8.72	4	5.0×10^{-5}
^{14}C -Testosterone	10	1.75	2	Below detection
^3H -Estradiol	10¶	14.68	2	Below detection
^3H -Cortisone	10¶	9.54	2	Below detection
^{14}C -DL-Glutamic acid	75	3.42	2	4.0×10^{-5}
^{14}C -L-Glutamic acid	75	111.22	2	4.0×10^{-5}

* Micrograms of "antigen" complexed per microgram of RNA per microgram of total antigen (intracellular or in homogenate). Corrected for controls (antigen and purified RNA mixed in phosphate buffer and subjected to the same extraction procedure).

‡ No difference was found between the alum-precipitated and the methylated bovine serum albumin-complexed forms (5).

§ Some experiments were done with mouse and guinea pig PE cells.

|| These experiments were done with PE cells from CBA and C57 mice.

¶ 1 part of radioactive compound was diluted with 100 parts of cold hormone on a weight basis.

form RNA complexes is expressed as the efficiency of binding, in terms of micrograms of antigen bound per microgram of RNA, divided by the total antigen taken up by the cells or introduced into the homogenate (9). ^3H -Poly-D-glutamic acid was the most extensively investigated molecule, being assayed in 44 separate experiments. It had an efficiency of binding range of $1.7-10.0 \times 10^{-4}$, about 2-fold higher on the average than ^3H -poly- α -D-glutamic acid or carboxylated ^{14}C -COOH-dextran and 10-fold greater than a human ^{125}I - γG myeloma protein or ^{125}I -(TGAL). The binding of the noncarboxylated ^3H -dextran was below detectable levels. There was no discernible distinction between the association of ^{125}I -(TGAL) with RNA from PE cells of strain CBA or C57 mice.

Of the low molecular weight compounds tested, only ^{14}C -glutamic acid showed detectable association with RNA, with an efficiency about equal to that of the human immunoglobulin and (TGAL). All the data are given in terms of weight rather than moles, because the molar ratios of components in the complexes are unknown. Since DL-glutamic acid and L-glutamic acid associated similarly, it is unlikely that a significant amount of binding was due to the loading of an

FIG. 1. Association of $250\ \mu\text{g}\ ^3\text{H}$ -poly- γ -D-glutamic acid (O), $250\ \mu\text{g}\ ^3\text{H}$ -poly- α -D-glutamic acid (∇), and $572\ \mu\text{g}\ ^{14}\text{COOH}$ -dextran (●) with various quantities of RNA. The "antigens" were incubated with different quantities of PE cell homogenate, and the quantities of RNA are values of RNA isolated following incubation.

aminoacyl-tRNA specific for glutamic acid. This was supported by *in vitro* experiments in the absence of aminoacyl-tRNA synthetase, which are described below. Complex formation was not detected with ^{14}C -testosterone, ^3H -estradiol, or ^3H -cortisone. Thus, of the molecules tested for capacity to associate with RNA, only those carrying negatively charged groups were found to do so.

The data plotted in Fig. 1 show that for a given amount of "antigen," association was proportional to the quantity of RNA. Although relatively few points were obtained for ^3H -poly- α -D-glutamic acid, both this compound and $^{14}\text{COOH}$ -dextran had a lower capacity for association on a weight basis than ^3H -poly- γ -D-glutamic acid. Thus, when $250\ \mu\text{g}$ of either polypeptide was used in a cell-free

system, the γ -polymer required about 165 μg of RNA for 15% binding, as compared to 600 μg for the α -polymer. While the ranges of association for the two polymers overlapped, as shown in Table I, poly- γ -D-glutamic acid was consistently more efficient when cells from the same animal were used in assays of the different compounds. Conversely, for a constant amount of RNA, the quantity of "antigen" bound was proportional to the total quantity used. It was not possible, within the practical limits of the assay system, to saturate RNA with antigen. The relationship between the quantity of antigen added and the amount bound to RNA remained essentially linear over the range tested. When mixtures of antigens were used, there was no demonstrable competition between them for the available RNA. For example, a 20-fold excess by

TABLE II
Association with RNA of $^{14}\text{COOH}$ -dextran Incubated with Peritoneal Exudate Cells for Various Time Periods

Duration of incubation*	Isolated RNA	Dextran/RNA ratio	Efficiency of binding†
	μg	$\mu\text{g}/\mu\text{g}$	
1 min	267	0.19	3.1×10^{-4}
10 min	337	0.16	2.6×10^{-4}
1 hr	372	0.23	3.8×10^{-4}
4 hr	442	0.12	1.9×10^{-4}
19 hr	423	0.15	2.4×10^{-4}
24 hr	447	0.11	1.7×10^{-4}
48 hr	185	0.26	4.3×10^{-4}

* 572 μg of $^{14}\text{COOH}$ -dextran incubated with homogenates of 10^8 cells at 37°C.

† Micrograms of ^{14}C -dextran complexed per microgram of RNA/total micrograms of ^{14}C -dextran, corrected for background.

weight of unlabeled DL-glutamic acid did not affect the association of a given quantity of ^3H -poly- γ -D-glutamic acid, nor did a smaller excess of unlabeled poly- γ -D-glutamic acid itself.

Variation of Conditions of Incubation with Antigen.—The length of time of incubation of $^{14}\text{COOH}$ -dextran with cell homogenates prior to extraction of RNA was varied from 1 min to 48 hr, without significant effect on binding (Table II). These experiments were performed using equal numbers of cells from the same animal.

Incubation with antigen was usually carried out at 37°C. When it was performed at 2°C, 20°C, or 37°C, or after heating the cell homogenate at 80°C for 5 min, no significant differences were found in antigen/RNA ratios or in efficiencies of binding for the two molecules, ^3H -poly- γ -D-glutamic acid and $^{14}\text{COOH}$ -dextran.

Cell homogenates subjected to treatment with Pronase or dithiothreitol prior to incubation with antigen, or incubated with antigen in the presence of 0.5% sodium dodecyl sulfate, were not demonstrably different from untreated

TABLE III
*Association with RNA of ^3H -Poly- γ -D-glutamic Acid Incubated with Peritoneal Exudate Cells at Various Temperatures**

Amount of ^3H -polypeptide	Temperature	Buffer	Isolated RNA	Polypeptide/ RNA ratio	Efficiency of binding†
μg	$^{\circ}\text{C}$		μg	$\mu\text{g}/\mu\text{g}$	
120	37	Phosphate§	383	0.025	2.1×10^{-4}
120	2	Phosphate	502	0.024	2.0×10^{-4}
120	80	Phosphate	436	0.023	1.9×10^{-4}
120	37	Acetate-SDS	324	0.038¶	3.2×10^{-4} ¶
240	37	Phosphate	581	0.041	1.7×10^{-4}
120 for 15 min, then 120 μg more	37	Phosphate	475	0.050	2.1×10^{-4}

* ^3H -Poly- γ -D-glutamic acid incubated during 30 min with homogenates of 10^8 cells from the same rabbit.

† Micrograms of ^3H -poly- γ -D-glutamic acid complexed per microgram of RNA/total micrograms of polypeptide added.

§ 0.015 M sodium phosphate, pH 7.4, and 0.14 M NaCl.

|| 0.01 M sodium acetate buffer (pH 5.0), 0.1 M NaCl, and 0.5% sodium dodecyl sulfate.

¶ The somewhat higher efficiency obtained in this buffer was not reproducible.

TABLE IV
*Association with RNA of $^{14}\text{COOH}$ -dextran after Treatment of the PE Cell Homogenate with a Proteolytic Enzyme, a Reducing Agent, or a Chelating Agent**

Cells	Isolated RNA	^{14}C -Dextran/RNA ratio	Efficiency of binding†
	μg	$\mu\text{g}/\mu\text{g}$	
Standard	224	0.20	3.3×10^{-4}
Pronase-treated	158	0.21	3.5×10^{-4}
Dithiothreitol-treated	137	0.22	3.6×10^{-4}
0.01 M EDTA	136	0.21	3.5×10^{-4}

* 572 μg of $^{14}\text{COOH}$ -dextran incubated at 37°C with homogenates of 10^8 cells from the same rabbit.

† Micrograms of ^{14}C -dextran complexed per microgram of RNA/total micrograms of ^{14}C -dextran.

homogenates in their capacity to complex with antigen. Furthermore, addition of antigen in one or several doses had no influence on the final result. Tables III and IV summarize some of the experimental results obtained with ^3H -poly- γ -D-glutamic acid and $^{14}\text{COOH}$ -dextran. Although not reflected by the figures in

Table IV, in some experiments up to a 50% diminution of binding was observed in the presence of 0.01 M EDTA.

These results suggest that the formation of RNA-antigen complexes is probably not an enzyme-mediated reaction.

RNA-Antigen Complex Formation and RNA Synthesis.—The incorporation of ^{14}C -uridine into RNA was determined for cells suspended in Eagle's minimal essential medium (MEM) and incubated with ^3H -poly- γ -D-glutamic acid, either complexed with methylated albumin or precipitated with alum or with saline. The MEM afforded more efficient uptake of antigen (30%) than the phosphate buffer used in earlier experiments (9), but, again, association was indistinguishable for the two forms of the polypeptide.

TABLE V
*Incorporation of ^{14}C -Uridine into RNA from Peritoneal Exudate Cells during Exposure to ^3H -Poly- γ -D-glutamic Acid Complexed with Methylated Albumin (MBSA) or Precipitated with Alum**

Treatment of cells	Isolated RNA	^{14}C	$^{14}\text{C}/\text{RNA}$	Efficiency of binding†
	μg	<i>cpm</i>	<i>cpm/\mu\text{g}</i>	
Saline	1,022	32,500	32	
^3H -Poly- γ -D-glutamic acid-alum	1,105	28,500	26	2.0×10^{-4}
^3H -Poly- γ -D-glutamic acid-MBSA	1,279	42,000	32	2.0×10^{-4}

* 10^9 cells incubated during 15 min with $310 \mu\text{g}$ ^3H -polypeptide and $5 \mu\text{C}$ ^{14}C -uridine.

† Micrograms of ^3H -poly- γ -D-glutamic acid complexed per microgram of RNA/micrograms of total ^3H -polypeptide.

The incorporation of ^{14}C -uridine into RNA was not significantly different in the absence or presence of the polypeptide in either of its two forms in the cell culture (Table V). In every case, sucrose density gradient ultracentrifugation showed that all the detectable ^{14}C and ^3H radioactivity was associated with the 4-5S RNA peak, confirming earlier results (9).

In experiments in which nuclei and mitochondria were removed from PE cell homogenates by centrifugation and the remaining fraction was incubated with polypeptide, the efficiency of binding to RNA was indistinguishable from experiments in which unfractionated homogenates were used. There was no incorporation of ^{14}C -uridine under those circumstances, establishing that RNA synthesis had not taken place. In other experiments, actinomycin D was used to block transcription, and, again, association of antigen with RNA was unimpaired (Table VI). A level of ^{14}C -uridine incorporation $1/1000$ that of the control could have been detected. These results make it extremely unlikely that RNA synthesized after the introduction of antigen is needed for the formation of complexes.

Association of "Antigen" with RNA from HeLa Cells.—When 117.5 μg of ^3H -poly- γ -D-glutamic acid was incubated for 30 min with a homogenate of 5×10^8 HeLa cells, 1254 μg of RNA were recovered. Polypeptide was found associated with this RNA, the polypeptide/RNA ratio being 0.048 and the efficiency of binding 4.1×10^{-4} . This compares very well with the values obtained using PE cells (Table I). Thus, complexes can form between antigen and RNA from cells other than macrophages, although under normal circumstances such cells are not phagocytic.

Distribution of "Antigen" in Subcellular Fractions.—Table VII shows the subcellular distribution in PE cells of ^3H -poly- γ -D-glutamic acid introduced as an electrostatic complex with methylated albumin or as an alum precipitate.

TABLE VI
*Incorporation of ^{14}C -Uridine into RNA from Peritoneal Exudate Cells during Exposure to ^3H -Poly- γ -D-glutamic Acid in the Presence or Absence of Actinomycin D**

Actinomycin D	Isolated RNA		Polypeptide/RNA ratio	Efficiency of binding†
	μg	<i>cpm</i>		
—	116	20,520	1.0×10^{-3}	5.0×10^{-4}
+	108	0	0.9×10^{-3}	5.0×10^{-4}

* 600 μg actinomycin D incubated with 10^8 cells 45 min prior to addition of 10 μC ^{14}C -uridine and 310 μg ^3H -poly- γ -D-glutamic acid.

† Micrograms of polypeptide complexed per microgram of RNA/micrograms of total polypeptide.

Although these experiments were crudely done, in that nuclei were not separated from intact cells or other cell debris and the dispersion of radioactivity due to lysosomal disruption was not investigated, the results suggest that there was no significant difference between the two forms in the proportions of total radiolabel in the soluble fraction, which contained most of the 4-5S RNA.

Formation of Complexes with Purified RNA.—The observations that ^3H -poly- γ -D-glutamic acid and other molecules formed very stable complexes only with 4-5S RNA, that they did not take place between antigen and purified RNA mixed in vitro (9), as well as the characteristics of complex formation established above, led to further studies based on reports of the conversion of an expanded form of tRNA to the compact coiled form by chelation of Mg^{++} ions, and the remarkable stability of the coiled form (15-18).

Table VIII shows the association between purified RNA from PE cells and some of the "antigens" used in these studies, in buffers with or without Mg^{++} ions. The dependence of complex formation on the presence of the divalent cation in the medium and anionic groups on the "antigen" is apparent. Efficiencies of association in the presence of Mg^{++} ions were comparable to those in

experiments in vivo, but the omission of Mg^{++} or its removal by excess EDTA held the radioactivity of precipitated RNA to background levels. Concentrations of Mg^{++} as low as 2×10^{-4} M were tested and proved fully effective (Table

TABLE VII
*Subcellular Localization in PE cells of 3H -Poly- γ -D-glutamic acid Precipitated with Alum or Complexed with Methylated Albumin (MBSA)**

Fraction	3H -Poly- γ -D-glutamic acid-alum radioactivity		3H -Poly- γ -D-glutamic acid-MBSA radioactivity	
	Total	Intracellular	Total	Intracellular
	dpm	% dpm	dpm	% dpm
I. Mitochondria-lysosomes	1,792,500	69	30,485	61
II. Endoplasmic reticulum-lipo-proteins	81,750	3	2,950	6
III. Ribosomes	24,310	1	4,700	9
IV. Soluble	712,250	28	12,125	24

* Incubation with 10^8 cells in MEM during 30 min at 37°C.

TABLE VIII
Formation of RNA-"Antigen" Complexes Using Purified RNA

"Antigen"	Buffer†	Temperature	RNA-associated antigen*		
			Radioactivity	Per cent	Efficiency
		°C	cpm		
3H -Poly- γ -D-glutamic acid	1	25	18,250	7	5.0×10^{-4}
	1	60	26,775	11	9.0×10^{-4}
	2	25	0	0	
	3	25	0	0	
	4	25	0	0	
	4	60	0	0	
^{14}C -DL-Glutamic acid	1	60	840	5	3.7×10^{-4}
^{14}C -DL-Glutamic Acid	2	60	0	0	
3H -Estradiol	1	60	0	0	
3H -Cortisone	1	60	0	0	
^{14}C -Testosterone	1	60	0	0	

* Corrected for controls (antigen in the absence of RNA < 0.5%).

† 1 = 0.02 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA, 0.01% sodium dodecyl sulfate; 2 = 1 without $MgCl_2$; 3 = 1 saturated with EDTA; 4 = 0.015 M sodium phosphate (pH 7.4) in 0.14 M NaCl.

IX). Under the conditions used, the dextrans precipitated in the absence of RNA and thus could not be assayed.

The sucrose density gradient pattern of complexes formed between purified RNA and 3H -poly- γ -D-glutamic acid in the presence of 0.02 M Mg^{++} (Fig. 2) revealed that most of the radiolabel sedimented with 4-5S RNA although

about 18% was associated with aggregated material at the bottom of the gradient, and 11–17% with 28S and 16S RNAs. Although the gradient was centrifuged for 3 hr instead of the customary 6 hr, it failed to reveal distinct peaks of labeled aggregated material. It has been shown that Mg^{++} ions will cause aggregation of RNA (19), presumably by producing intermolecular chelates, and the conditions of these experiments did not simulate those using viable cells or cell homogenates, which probably accounts for the differences in distribution of radioactivity in the gradients.

TABLE IX
Formation of Complexes between 3H -Poly- γ -D-glutamic Acid and Purified PE Cell RNA at Different Mg^{++} Concentrations

[Mg^{++}]*	Radioactivity in complex†	
	Total	Per cent
<i>M</i>	<i>cpm</i>	
0.02	16,010	15
0.004	23,970	22
0.002	22,056	21
0.0002	22,814	21

* As $MgCl_2$, in 0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA, and 0.01% sodium dodecyl sulfate.

† Corrected for controls (antigen in the complex in the absence of Mg^{++} < 1%).

Recalling the behavior of tRNA (17, 20), dissociation of RNA-polypeptide complexes formed in vivo was achieved by exhaustive dialysis against a buffer containing 0.028 M EDTA (Table X). Although at 20°C there was some dissociation, 80°C, a temperature at which tRNA melts (21), proved far more effective. Even at the higher temperature, dissociation did not take place in the absence of EDTA (Table X).

DISCUSSION

The object of this investigation was to assess the relevance of macrophage RNA-antigen complexes to the induction of the immune response. Toward that end, the specificity of a number of facets of the phenomenon were investigated, including the "antigens," the RNAs, the involvement of specific enzyme(s), and the requirement for a specific type of cell (the macrophage). The evidence demonstrates a lack of specificity at every level and strongly indicates that the RNA-"antigen" complex is a chelate formed between divalent cations and anionic groups on the components of the complex.

If the binding of antigen to macrophage RNA is a crucial early step in immune induction, some degree of differentiation between molecules on the basis of immunogenicity might be anticipated, particularly if the macrophage is con-

sidered the antigen-recognizing cell (1, 22). The evidence presented here is in substantial disagreement with such a hypothesis. In over 40 experiments, poly- γ -D-glutamic acid, whether precipitated with alum or complexed with methylated albumin, behaved indistinguishably with respect to capacity to associate with RNA. In the former form, the polypeptide is at best a very weak

FIG. 2. Sucrose density gradient ultracentrifugation of ^3H -poly- γ -D-glutamic acid complexed in vitro with purified RNA. The 10–40% gradient was centrifuged for 3 hr at 140,000 g in a type SW 41 rotor. Because of the short period of centrifugation used to disclose the distribution of radioactivity in aggregates of RNA, the 28S, 16S, and 4S RNA peaks are compressed near the top of the gradient.

immunogen, if not totally nonimmunogenic, in rabbits. Serum antibodies could not be detected in rabbits injected repeatedly with doses ranging from 10 μ g to 10 mg (11), nor with multiple simultaneous intradermal injections (11), which have been used to elicit weak responses to a polypeptide previously thought to be nonimmunogenic (23, 24). The albumin complex, on the other hand, regularly elicited substantial quantities of antibody which reacted with the polypeptide itself (10). In animals producing antipolypeptide antibody, the polypeptide was unable to serve as a carrier for a conjugated azohapten (10), nor could it elicit delayed skin reactions, in contrast to the total complex or its albumin component (11). An analogous situation has been described for the

TABLE X
Dissociation of a ^3H -Poly- γ -D-glutamic Acid-RNA Complex Obtained In Vivo

Dialysis	Buffer*	Temperature	Polypeptide/RNA ratio (% of starting complex)
			$\mu\text{g}/\mu\text{g}$
—	1	20	100
+‡	1	20	69
—	1	80	100
+	1	80	31
—	2	20	95
+	2	80	100

* 1 = 0.02 M Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate, and 0.028 M EDTA; 2 = 1 without EDTA.

‡ The complexes were suspended in a 1 ml volume and dialyzed for 3 days against 250 ml of buffer, changed twice daily.

behavior of responder and nonresponder guinea pigs to 2,4-dinitrophenylpoly-L-lysine (25).

There is additional evidence which conflicts with a relationship between immunogenicity and capacity to form complexes with RNA. Whereas pure polysaccharides are nonimmunogenic in rabbits (26), $^{14}\text{COOH}$ -dextran showed appreciable binding with RNA while ^3H -dextran did not. Mice of strain C57 respond well to the synthetic polypeptide (TGAL), while CBA mice respond poorly (27). This polypeptide showed the same degree of association with RNA when incubated with PE cells from either of the two strains. Glutamic acid is neither immunogenic nor a complete antigenic determinant, but shows an association comparable to that given by human γG immunoglobulin. The steroids, which may serve as haptens (28), were negative in association assays.

Thus, molecular size and chemical complexity do not appear to be factors in binding with RNA, although they do seem to be crucial for immune induction, as illustrated, for example, with a series of α -dinitrophenyloligolysine peptides

(29, 30). The single property which all the binders share and the nonbinders lack is the possession of anionic groups. Based on the absence of a correlation between the binding molecules and immunogenicity, it could still be postulated that binding to macrophage RNA is a necessary but insufficient event for the immune response. However, the absence of binding, within the limits of detection, of molecules which lack negative charges vitiates this contention.

There may even be a correlation between the pK of the carboxyl groups on the "antigen" and their efficiency of binding to RNA, although the evidence is too thin to establish this unequivocally. The free α -carboxyl groups of the γ -glutamyl polypeptide have a pK of 3.6 ± 0.1 in 1 N KCl at 25°C (31), while the γ -carboxyls of the α -polypeptide have a pK of about 5.0 (32). The former polypeptide is a more efficient binder than the latter (Fig. 1).

The kinetics of association excluded the involvement of an enzyme and hence rendered unlikely covalent binding between RNA and "antigen." Varying the times of incubation from 1 min to 48 hr, the temperature from 2°C to 37°C, heating the cell homogenate to 80°C or treating it with Pronase or dithiothreitol prior to incubation with "antigen," or incubating the components in the presence of a dispersing agent such as sodium dodecyl sulfate exerted no influence on the formation of complexes.

The observations that RNA could not be saturated by increasing the quantity of "antigen" within reasonable limits, that newly synthesized RNA was not required for complex formation, and that association occurred as readily with homogenates of HeLa cells as with PE cells all attest to the absence of unique species of RNAs in this phenomenon. In agreement with this conclusion, hybridization experiments showed that RNA from macrophages incubated with an antigen was indistinguishable from the RNA of normal macrophages (33, 34).

The identical incorporation of ^{14}C -uridine found whether PE cells were exposed to saline or to poly- γ -D-glutamic acid, alone or complexed with methylated albumin, appears to conflict with a report that such cells incubated with sheep erythrocytes synthesized appreciably larger amounts of low molecular weight RNA than did controls (35). However, no relationship was shown between RNA synthesis and immunogenicity of the erythrocytes, and a higher rate of RNA synthesis might be expected of cells actively processing such a complex material. On the other hand, the present results indicate that immunogenicity per se does not perceptibly alter macrophage RNA synthesis.

The nature of the bond between 4-5S RNA and "antigens" with anionic groups remained an intriguing puzzle. It seemed curious to find two negatively charged molecules joined noncovalently, yet able to unite only in the presence of some vital cellular component. Some recently disclosed properties of tRNA provided important clues. tRNA exists in essentially two forms in the cytoplasm. A more open or expanded form, considered a precursor, can be trans-

formed to the mature coiled form by Mg^{++} bridges between phosphate residues in the RNA molecule (15, 16, 36-38); the coiled form is extremely stable (17, 18). High concentrations of Mg^{++} cause aggregation of tRNA, presumably through the formation of intermolecular chelates (15). These findings, coupled with the characteristics of RNA-antigen complex formation, suggested that chelation of metal ions by anionic groups could account for the formation of these complexes, the vital cellular component being divalent cations. This was borne out by implementing the complexing of pure RNA and "antigen" by the addition of magnesium salts (Table IX) and effecting the dissociation of complexes formed *in vivo* by exhaustive dialysis against buffers containing a chelating agent (Table X). The effectiveness of a magnesium concentration as low as $2 \times 10^{-4} M$ and the necessity for high concentrations of EDTA to abolish the effect suggest that few bonds are needed to form a complex. The remarkable stability of the complexes suggests that they may be stabilized by a conformational change analogous to the maturation of tRNA.

It should be strongly emphasized that formation of RNA-antigen complexes within viable cells has never been demonstrated. A recent report indicated that small pinocytosed peptides were stored in lysosomes and that molecules larger than di- or tripeptides were unlikely to escape to the cytoplasm of the macrophage (39). The RNA extraction procedure involves disrupting the organization of the cell, and it seems probable that the complexes seen are an artifact of this procedure. The enhanced immunopotency of complexes obtained after extraction of RNA (8) is not a unique phenomenon; linking antigens to bentonite, for example, also intensifies the antibody response (40).

What, then, can be said for the role of the macrophage in immune induction? In addition to macrophage RNA-antigen complexes, theories postulating a crucial role for macrophage RNA have proposed the transfer to lymphoid cells of a functional antigen-free messenger RNA (41). Askonas et al. (5) have recently reviewed the numerous convincing arguments against an important physiological role for the transfer of a messenger RNA which codes for specificity and allotypic sequences of immunoglobulins. The report that the precursor of the antibody-forming cell, produced in the bone marrow, is induced to active synthesis by an antigen-reactive cell, also of the lymphoid type but from the thymus (42-44), also argues against a direct transfer of information from the macrophage to the immunocyte. The association of a fraction of antigen with macrophage cell membranes and the increased immunopotency of antigen in that form may indicate that, after all, the role of the macrophage in immune induction is not to "process" the antigen, but to expose it in a more effective way to lymphoid cells (45-48).

SUMMARY

10 different compounds, including natural and synthetic polypeptides, proteins, polysaccharides, amino acids, and steroid hormones, were assayed for

their capacity to form complexes with peritoneal exudate cell RNA. Only molecules carrying negatively charged groups were able to do so.

The formation of RNA-antigen complexes was unrelated to the immunopotency of the "antigen," was not an enzyme-dependent reaction, did not require the synthesis of RNA following introduction of the antigen, did not seem to involve antigen-specific RNAs, was not specific for macrophages, since HeLa cells could be used as effectively, and occurred when purified RNA was mixed with antigen only in the presence of divalent cations. The complexes were very stable, once formed, but could be dissociated by exhaustive dialysis against buffers containing a chelating agent.

The macrophage RNA-antigen complex therefore appears to be a chelate between anionic groups on the two components. Based on the total absence of a relationship between immunogenicity and the capacity to form such complexes, as well as the nonspecific nature of complex formation at every level examined, it appears unlikely that RNA-antigen complexes play a physiologically significant role in immune induction.

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