Effect of Trypsin and Ribonuclease on the Immunogenic Activity of Ribosomes and Ribonucleic Acid Isolated from *Mycobacterium tuberculosis*

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ABSTRACT

YOUMANS, ANNE S. (Northwestern University Medical School, Chicago, Ill.), AND GUY P. YOUMANS. Effect of trypsin and ribonuclease on the immunogenic activity of ribosomes and ribonucleic acid isolated from *Mycobacterium tuberculosis*. J. Bacteriol. 91:2146-2154. 1966.—The ribosomal fraction of the attenuated strain, H37Ra, of *Mycobacterium tuberculosis* was treated with trypsin alone, ethylenediaminetetraacetic acid (EDTA) alone, EDTA and pancreatic ribonuclease, or with trypsin and ribonuclease. After each of these treatments, the ribosomal fractions were injected intraperitoneally into male CF-1 mice to test their capacity to produce an immune response to infection with virulent tubercle bacilli, strain H37Rv. Removal of protein with trypsin left the immunogenicity unchanged; EDTA alone reduced immunogenicity in the smaller vaccinating doses; EDTA plus ribonuclease reduced the immunogenicity by approximately 50% in the highest (1.0 mg) vaccinating dose; ribonuclease alone, after treatment with trypsin, reduced immunogenicity also approximately 50%. A crude mycobacterial ribonucleic acid (RNA) was prepared by extraction of the ribosomal fraction with alcohol. This RNA preparation was as effective in producing an immune response as the ribosomal fraction from which it was prepared, unless the RNA was partially or completely degraded during the preparation. The effect of ribonuclease on the immunogenicity of the RNA was similar to that obtained with the ribosomal fractions, except that ribonuclease completely destroyed the immunogenicity of a partially degraded RNA. RNA appears to be an essential part of an immunizing substance in attenuated tubercle bacilli, which produces a high degree of immunity in mice; 50 μ g (dry weight) will protect approximately 80% of the mice, and as little as 0.5 μ g will protect approximately 30% of the mice. Mycobacterial RNA not incorporated in Freund's incomplete adjuvant was nonimmunogenic. Yeast RNA incorporated in Freund's incomplete adjuvant was not immunogenic.

In the previous paper (37), methods were described in which sodium dodecyl sulfate (SDS) was used to prepare mycobacterial ribosomal fractions. These fractions were immunogenic in mice when administered in much smaller vaccinating doses than had been found to be active previously (33-36). Having obtained more active and more stable ribosomal preparations, experiments were devised to determine whether the entire ribosomal particle was needed to produce the immune response or whether one of its two components, ribonucleic acid (RNA) or protein, was the immunizing moiety. The re-

sults suggest that RNA is the immunogenic substance.

MATERIALS AND METHODS

The methods and materials, including the preparation of the SDS ribosomal fraction, were the same as those given in the previous paper. Certain other experimental details will be given with the results. All vaccine preparations were administered to mice in Freund's incomplete adjuvant unless otherwise noted.

RESULTS

To help define the role of protein in the immunogenic process, crystalline trypsin was added

to a portion of the ribosomal preparation (0.05 mg/mg of ribosomal fraction, pH 7.0) and incubated at 37 C for 30 min. This amount of trypsin was added to the phosphate-magnesium buffer which served as the blank. Protein was determined by the method of Lowry et al. (14), and the average amount for the preparations is given in Table 1. Mice were vaccinated with different amounts of each preparation, and the pooled data of three to five separate experiments are shown in Table 1.

Trypsin-treated and nontreated ribosomal material immunized mice to the same degree, except in the 0.1-mg vaccinating dose. However, in two of the five individual experiments, the trypsin-treated material was as immunogenic as the control nontrypsinized material, or more immunogenic than the control. These results show that protein is not necessary for the immunogenic activity of the ribosomal fraction.

Experiments were done with crystalline pancreatic ribonuclease (protease-free; Mann Research Laboratories, New York, N.Y.) to determine whether ribonuclease would lower the immunogenic activity of the ribosomal fraction. We had shown earlier (35) that the presence of ribonuclease could significantly decrease the immunogenic activity of the particulate fraction, but, because of the large amounts of ribonuclease needed, and the relatively low degree of immunogenic activity of the control particulate fractions, more definitive experiments were needed. In this series of experiments, the treatment of the ribosomal fractions with ribonuclease was varied in the hope of finding the optimal conditions for ribonuclease activity.

The first experiment with ribonuclease was done at 4 C, since earlier studies had shown that,

for maximal immunogenic activity, the immunizing fraction had to be kept cold. Since the enzymatic activity of ribonuclease at that temperature would be low, an equal volume of a 4% concentration of ethylenediaminetetraacetic acid (EDTA) was added to the ribosomal fraction (200 mg/ml). A portion was removed, and crystalline pancreatic ribonuclease was added (1 mg/mg of ribosomal fraction), the mixtures were allowed to stand overnight at 4 C, and the treated fractions were recentrifuged at $144,700 \times g$ for 3 hr. The EDTA was added to serve as a chelating agent to remove the magnesium ions from the ribosomal particles; this, in turn, should affect adversely the stability of the ribosomes (3, 15, 16, 27, 30) and possibly make the RNA more susceptible to the action of ribonuclease. It also has been shown (13, 29) that magnesium ions may inhibit ribonuclease activity. The data from one experiment are given in Table 2. Ribonuclease, under these conditions, reduced the immunogenic activity of the 1.0-mg vaccinating dose of the EDTA-treated ribosomal fraction by 37%. It was found, however, that the presence of EDTA alone eliminated the immunogenic activity of the lower vaccinating dose.

Since ribonuclease reduced the immunogenic activity in this experiment by only 37%, and in view of the finding that the ribosomal fraction appears fairly stable at 37 C for 30 min (Table 1), additional experiments were done in which ribonuclease was added to the ribosomal particles at 37 C.

To a ribosomal fraction (100 mg/ml) was added EDTA (final concentration, 2%) and ribonuclease (1 mg/mg of ribosomal fraction). The mixture was incubated in a water bath at 37 C for 30 min. The results are shown in Table

TABLE 1. *Effect of trypsin on the immunogenic activity of the ribosomal fraction*

Immunizing prepn	Amt injected (mg)*	No. of mice	No. of S-30 mice†	Per cent of S-30 mice	Amt (μg) of protein/mg* of immunizing prepn
Ribosomal fraction	20.0	67	56	84	35
	1.0	142	97	69	
	0.1	130	78	60	
Ribosomal fraction treated with trypsin	20.0	66	51	77	0
	1.0	114	89	78	
	0.1	140	59	42	
H37Ra	1.0	140	132	94	
Controls		144	24	17	

* Moist weight.

† S-30 mice = number of mice which survived >30 days.

TABLE 2. *Effect of EDTA and ribonuclease at 4 C on the immunogenic activity of the ribosomal fraction*

Immunizing prepn	Amt injected (mg)*	No. of mice	No. of S-30 mice†	Per cent of S-30 mice
Ribosomal fraction	1.0	30	26	87
	0.1	30	26	87
Ribosomal fraction treated with EDTA	1.0	29	26	90
	0.1	30	4	13
Ribosomal fraction treated with EDTA and ribonuclease	1.0	30	17	57
	0.1	28	1	4
H37Ra	1.0	30	30	100
Controls		28	1	4

* Moist weight.

† S-30 mice = number of mice which survived >30 days.

TABLE 3. *Effect of EDTA and ribonuclease at 37 C on the immunogenic activity of the ribosomal fraction*

Immunizing prepn	Amt injected (mg)*	No. of mice	No. of S-30 mice†	Per cent of S-30 mice
Ribosomal fraction at 0 C	1.0	27	19	70
	0.1	26	11	42
Ribosomal fraction treated with EDTA	1.0	30	25	83
	0.1	26	1	4
Ribosomal fraction treated with EDTA and ribonuclease	1.0	30	12	40
	0.1	30	1	3
H37Ra	1.0	30	27	90
Controls		30	0	0

* Moist weight.

† S-30 mice = number of mice which survived >30 days.

3. The presence of ribonuclease under these conditions decreased the immunogenic activity of the 1.0-mg vaccinating dose by 51%. Although the ribonuclease was more active at 37 C than at 4 C, the results of the experiments at the two temperatures were similar. The presence of EDTA again eliminated the immunogenic response obtained in the mice vaccinated with 0.1 mg.

In another experiment, the ribosomal fraction was treated with trypsin at 37 C for 30 min to remove ribosomal protein, and then ribonuclease was added and the incubation was continued for 30 min. Ribonuclease decreased the number of survivors from 75 to 35% in the mice vaccinated with the 1-mg dose, a reduction of 51%. Of the mice given the 0.1-mg dose, 61% survived in comparison with 38% which survived when given the same amount of ribonuclease-treated material, a reduction of 38%.

Thus, a fairly large amount of ribonuclease was able to destroy only about 50% of the immunogenic activity. As a result, in the next five experiments a crude preparation of mycobacterial RNA was used to determine the activity of ribonuclease on mycobacterial RNA, and also to determine the immunogenic activity of this RNA.

The method used to extract the RNA followed, in part, the method devised by Crestfield, Smith, and Allen (4) for the isolation of RNA from yeast. The ribosomal fraction (20 mg/ml) was added to 2 volumes of cold 95% ethyl alcohol. To achieve precipitation of the RNA, it was necessary to add 2 M NaCl. The amount added

varied from 10 to 12 drops for a small volume, such as 30 ml, to several ml for a larger volume, but was sufficient to produce a gel-like flocculation upon standing at 0 C for approximately 30 min. This material then was centrifuged in the cold at 2,000 rev/min for 15 to 20 min. The supernatant fluid was discarded from the tightly packed gel-type white precipitate. The precipitate was suspended in distilled water (pH 7.0), and a Teflon grinder was used, when necessary, to gently homogenize the material. It was found that the precipitate could not be weighed accurately, as it apparently absorbed moisture during the alcohol extraction and was heavier than the original. The precipitate, therefore, was diluted to half the original volume, since experience showed that approximately half of the RNA was lost. The amount of RNA was measured by ultraviolet absorption. It was then diluted with distilled water so that the amount of RNA present was the same as the RNA content of the ribosomal fraction from which it was extracted.

The UV spectrum of this mycobacterial RNA was similar to spectra found with RNA extracted from other sources. The peak absorption was at 258 m μ . The 260:280 ratio was 1.8. These preparations of RNA were unstable at 37 C for 30 min, since after this time only acid-soluble fragments remained.

Dry weights were determined on samples of the crude RNA in four of the five experiments (Table 4). The amount of RNA averaged 2.1 mg/ml and protein averaged 1.2 mg/ml; there-

TABLE 4. *Amounts of RNA and protein in mycobacterial RNA preparations*

RNA prepn	Dry wt	RNA	Protein	RNA-protein ratio	Per cent dry weight, RNA + protein
	mg	mg	mg		
1	1	0.77	0.16	4.8	93
2	1	0.51	0.57	0.89	108
3		1.14	0.55	2.07	
4	1	0.30	0.14	2.1	44
5	1	0.38	0.18	2.1	56

fore, RNA constituted about 64% of the RNA plus protein weight. In two of the four experiments, RNA plus protein were almost equal to the dry weight. The dry weight was approximately one-twentieth of the wet weight.

These crude RNA preparations were injected into mice to determine immunogenicity. It was found that the results in the five experiments varied so that not all of the data could be pooled; however, for the comparison of the immunogenic activities of the RNA preparations with the ribosomal fractions from which the RNA was extracted, it was possible to pool the data from three experiments, and these are shown in Table 5. There was no significant difference in the immune response obtained in the mice vaccinated with dilutions of RNA and its control ribosomal fraction. Thus, this type of RNA immunized as well as did the ribosomal fraction. The amount of RNA injected was approximately the same in the crude RNA preparation as in the ribosomal fraction (40 to 50 μ g/mg, depending on the ribosomal preparation). The mycobacterial RNA

TABLE 5. *Comparison of the immunogenic activities of mycobacterial RNA and the ribosomal fraction from which it was extracted*

Immunizing prepn	Amt injected (mg)		No. of mice	No. of S-30 mice*	Per cent of S-30 mice
	Moist wt	Dry wt			
Ribosomal fraction	1.0	0.1	80	59	74
	0.1	0.01	77	37	48
	0.01	0.001	89	22	25
RNA	1.0	0.05	77	55	71
	0.1	0.005	77	32	42
	0.01	0.0005	89	24	27
H37Ra	1.0		90	73	81
Controls			90	2	2

* S-30 mice = number of mice which survived >30 days.

not incorporated into Freund's incomplete adjuvant produced no immune response.

The results of one experiment were not included in this table, because the mycobacterial RNA did not immunize as well as the ribosomal fraction from which it was extracted, although the amount of RNA in each preparation was the same (45 μ g/mg). These data are shown in Table 6. It was found that there was a correlation between the low immunizing activity and the amount of RNA degradation as measured by the absorption increment. The procedure for measuring the absorption increment followed that given by Hotchkiss (11). A portion of the mycobacterial RNA (1 mg/ml) was divided into two equal volumes (4.0 ml), and to one was added ribonuclease (0.1 mg/mg RNA). The same amount of ribonuclease was added to the same volume of distilled water to serve as a blank. The three mixtures stood at room temperature for 15 min, and the optical densities were then measured at 260 m μ . The difference obtained between the readings was divided by the original RNA reading to give the per cent increase in absorption increment. The increase for this preparation of RNA was only 10.8%; this indicated considerable degradation of the nucleic acid during its extraction. This finding was in contrast to the other three preparations of RNA given in Table 3, in which the average per cent increase in the absorption increment was 39%. This latter figure denotes RNA of good quality with little or no degradation (11). These results suggest, there-

TABLE 6. *Immunogenic activities of a partially degraded RNA and the ribosomal fraction from which it was extracted*

Immunizing prepn	Amount injected (mg)*	No. of mice	No. of S-30 mice†	Per cent of S-30 mice
Ribosomal fraction	1.0	30	19	63
	0.1	29	6	2
RNA	1.0	30	11	37
	0.1	30	1	3
RNA (24 C, 30 min)	1.0	29	12	41
	0.1	30	0	0
RNA (24 C, 30 min + ribonuclease)	1.0	30	2	7
	1.0	30	1	3
H37Ra	1.0	29	16	55
Controls		30	0	0

* Moist weight.

† S-30 mice = number of mice which survived >30 days.

fore, that the quality as well as the quantity of the RNA is important in the production of an immune response. The ribosomal fraction and whole cells were not as immunogenic as usual in this experiment. The results with the viable cells and the controls indicate that the infection was more severe.

In the first experiment in which RNA was prepared, it was found that this preparation of mycobacterial RNA, in contrast to the ribosomal fractions, was not stable at 37 C for 30 min. The RNA, at this temperature, broke down completely into acid-soluble fragments. Mice vaccinated with RNA treated at 37 C (with or without the addition of ribonuclease) showed no immune response, although the RNA kept at 0 C was as immunogenic as the ribosomal fraction from which it was extracted. These results again indicated that the RNA must be of high quality with little or no degradation in order to achieve a maximal immune response.

In two of the experiments, ribonuclease (0.1 mg/mg of RNA) was added to a portion of the RNA and allowed to stand at room temperature for 30 min, since the RNA appeared to be quite stable under these conditions. A room temperature control RNA sample and a 0 C control RNA sample were included also. After standing, the room temperature preparations were immersed in ice, and a portion was removed to test for the presence of acid-insoluble nucleic acid in order to determine the activity of ribonuclease under these conditions. It was found in the two experiments that ribonuclease decreased the acid-insoluble material by 50%, indicating that pancreatic ribonuclease does not hydrolyze completely mycobacterial RNA.

The animal experiments in which the mice were vaccinated with these preparations confirmed the chemical data in several ways. Since different results were obtained with these two ribonuclease-treated preparations, the data from each experiment are given separately. The data in Table 6 show that ribonuclease completely removed the immunogenic activity of the partially degraded RNA. As shown in Table 7, however, with RNA preparations of good quality (absorption increment of 40%), ribonuclease decreased the immunogenic activity of the 1.0-mg vaccinating dose by 45% and the 0.1-mg vaccinating dose by 49%. The mice vaccinated with the 0.01-mg dose of the ribonuclease-treated material were not significantly different from the controls.

These results show that there is a good correlation between the immunogenic response and the presence of acid-insoluble RNA; that the control RNA preparations held at room tem-

TABLE 7. *Effect of ribonuclease on the immunogenic activity of mycobacterial RNA*

Immunizing prepn	Amt injected (mg)*	No. of mice	No of S-30 mice†	Per cent of S-30 mice
RNA	1.0	30	21	70
	0.1	30	13	43
	0.01	30	8	27
RNA (24 C, 30 min)	1.0	30	22	73
	0.1	30	11	37
	0.01	30	7	23
RNA (24 C, 30 min + ribonuclease)	1.0	30	12	40
	0.1	21	4	19
	0.01	30	4	13
H37Ra	1.0	30	28	93
Controls		30	0	0

* Moist weight.

† S-30 mice = number of mice which survived >30 days.

perature for 30 min show little or no increase in acid-soluble fragments, that these preparations immunize the mice as well as the RNA preparations held at 0 C; and that RNA kept at 37 C for 30 min spontaneously became degraded into acid-soluble fragments which did not immunize mice.

Chemically, therefore, one possibly may be able to determine in advance whether an RNA preparation will be highly immunogenic by: (i) measuring the native quality of the RNA by obtaining the per cent absorption increment or (ii) by measuring the amount of acid-soluble fragments, or by use of both methods.

Finally, an experiment was done to determine the specificity of RNA. Yeast RNA in a 20-mg dose, incorporated into Freund's incomplete adjuvant, was used to immunize mice, and these mice were challenged in the usual fashion with the virulent H37Rv strain of *Mycobacterium tuberculosis*. Yeast RNA did not protect the mice against tuberculosis, since no mice were living at the end of the 30-day period.

DISCUSSION

These results strongly suggest that RNA is the labile entity in *M. tuberculosis* which produces the high degree of immunity against tuberculosis in the mouse. Trypsin, which destroyed the ribosomal protein, had no effect on the immunogenic activity of the ribosomes, showing that not only is protein not involved in immunogenicity but that the complete ribosomal

particle is not necessary for immunogenic activity.

The studies done to determine the effect of ribonuclease upon the immunogenic activity of the ribosomes and upon RNA extracted from the ribosomes revealed that only approximately 50% of the immunogenic activity was lost. This could be accounted for by the presence of a ribonuclease-resistant RNA core which still has the capacity to immunize. Pancreatic ribonuclease will hydrolyze pyrimidine-phosphate ester bonds but not purine-phosphate ester bonds. The ratio of purine to pyrimidine in RNA extracted from *M. bovis* (strain BCG) is 1.25 (24) or 1.08 (28), and possibly the same in the human, H37Ra strain, so there might be a purine polynucleotide core which would remain unhydrolyzed. In addition, Roth and Milstein (19) found that small quantities of detergent will inhibit ribonuclease activity, and the ribosomal preparations used in this study all had small amounts of SDS present. Attardi and Smith (2) and Pirie (17) suggested that the presence of protein, or a protein coat, may prevent ribonuclease from hydrolyzing poliomyelitis virus RNA. Other investigators (16, 27) also have found ribosomal RNA to be somewhat refractory to the enzymatic activity of pancreatic ribonuclease. It may be that, with purer preparations of mycobacterial RNA, ribonuclease may be more active. It should be added that in a few experiments in which the largest amount of ribonuclease (1.0 mg) employed has been incorporated alone into Freund's incomplete adjuvant and injected intraperitoneally into mice, a low-grade, nonspecific increase in resistance (20 to 25% survival) to tuberculous infection has been noted. This would be insufficient to account for all of the residual immunizing activity following ribonuclease treatment of the ribosomal fractions, and, in the experiments using RNA, the amounts of ribonuclease employed were too small to produce any such response alone.

However, the 50% reduction in immunogenic activity by ribonuclease is of greater significance than may be immediately apparent. It should be realized that a 10-fold dilution of the ribosomal fraction will decrease the immunogenic activity by only about 30%, and a 100-fold dilution of the ribosomal fraction will decrease the immunogenic activity by only about 60%. Therefore, the 50% reduction produced by ribonuclease may represent the hydrolysis of the major portion of the mycobacterial RNA.

Of particular significance were the findings that if the RNA became degraded during preparation, or by exposure to higher temperatures, the immunogenic activity also was decreased or destroyed. For example, all the activity was lost

by treatment at 37 C, and the RNA was found to be degraded completely into acid-soluble fragments. This demonstrated not only that the acid-soluble fragments are not immunogenic, but that mycobacterial RNA can be degraded by means other than the action of endogenous ribonuclease; this has been noted with RNA from other sources (9, 21, 29).

EDTA apparently had no effect on the ribonuclease activity, even though it may have removed the magnesium ions. These ions have been reported to be inhibitory to the enzymatic action of ribonuclease (13, 17, 20). However, EDTA alone, when added to the ribosomal fraction, reduced the immunogenic activity. No acid-soluble fragments were produced, though, after 30 min of exposure to this agent at room temperature or at 37 C. Palade and Siekevitz (16) found that EDTA damaged ribosomal particles from liver microsomes, resulting in a loss of 60 to 70% of the RNA. Chao (3) reported that EDTA dissociated yeast ribosomes, and Madison and Dickman (15) found aggregation of beef pancreas ribosomes in the presence of EDTA. The effect of EDTA on the ribosomes extracted from *Escherichia coli* and *Pseudomonas fluorescens* was studied by Wade and Robinson (31), and they found a marked difference between the two strains, dependent on the presence of endogenous ribonuclease.

The role of Freund's incomplete adjuvant is not clear, but it would be reasonable to assume that its major action is protective, preventing the RNA from being rapidly destroyed by host ribonuclease immediately after injection. Roth and Milstein (19) examined the ribonuclease content of rat tissues and found it present in all tissues examined, including serum. Since ribonuclease is present throughout the host, RNA injected alone would probably be degraded rapidly and would not produce an immune response, thus accounting for the lack of immunogenicity of mycobacterial RNA when not incorporated in adjuvant. Schwartz and Rieke (22) have shown that radioactive macromolecular RNA was incorporated into mouse macrophages after intraperitoneal injection. However, there was evidence that the RNA was partially degraded either before or shortly after it was taken up by the cells. Sved (26) has shown that RNA is rapidly hydrolyzed in the mouse when injected intravenously. In addition, Herriott, Connolly, and Gupta (10) and Alexander et al. (1) have shown that normal sera contain ribonuclease which destroys biologically active poliovirus RNA. Therefore, the heavy adjuvant would provide a protective coating for the RNA until it is taken up by macrophages, and perhaps for some time after ingestion.

The cell walls of whole cells, or the natural membranous adjuvant present in the particulate fraction, no doubt would protect the RNA in a similar fashion.

The mechanism whereby mycobacterial RNA might induce an immune response against tuberculosis in the mouse also is not known. Certainly, however, the RNA must provide a stimulus which in some manner eventually results in the proliferation of a population of macrophages which have a greater capacity to inhibit the multiplication of virulent tubercle bacilli. One can only speculate on how this might occur. First, an antibody to RNA might be formed which in turn would have the capacity to inhibit the multiplication of virulent tubercle bacilli. The absence of a circulating protective antibody against *M. tuberculosis* in immunized animals is well documented (18, 25), although Jenkin and Rowley (12) have suggested that a cell-bound antibody might be involved. Even if an antibody should be produced against RNA, it is difficult to visualize how this could combine with and affect the RNA inside of a living tubercle bacillus.

Second, mycobacterial RNA might, in some unknown fashion, stimulate the production of an adaptive enzyme or other substance which would have an inhibitory effect on mycobacterial growth. In regard to the latter possibility, Youmans and Youmans (32, 38) reported that a substance, which we called mycosuppressin, had been detected in the lungs of immunized guinea pigs and rabbits. Mycosuppressin inhibited the respiration and growth of virulent tubercle bacilli both in vitro and in vivo, and did not affect unrelated microorganisms. The nature of mycosuppressin is unknown, but it may be an adaptive enzyme, or it may be a substance normally present in the tissues, which increases greatly in amount after immunization. Conceivably, mycobacterial RNA might stimulate an overproduction of ribonuclease which would then function to inhibit growth. In this connection, Enright, Frye, and Atwall (5) showed an increase in ribonuclease in mice refractory to rabies, and suggested that the refractory state might be accounted for by the inactivation of the viral RNA by ribonuclease.

Third, even though the mycobacterial immunizing material is composed largely of RNA, and the immunizing activity is decreased by ribonuclease, there is a possibility that the RNA is only indirectly involved. Fishman (6) and Fishman and Adler (7) have shown that RNA from macrophages exposed to T2 bacteriophage would transfer to rat lymph node cells the capacity to

form antibodies against bacteriophage T2 antigens. The capacity of macrophage RNA to confer antibody-producing properties on lymph node cells was abolished by treatment with ribonuclease. More recently, Friedman, Stavitsky, and Solomon (8) showed that such macrophage RNA preparations always contain small amounts of T2 antigens. They also found that the transfer of capacity to produce antibody was greatly reduced after treatment of the macrophage RNA preparations with ribonuclease. They suggested that the macrophages rapidly degrade the T2 antigen and then bind the antigen fragments to certain low molecular weight RNA molecules of the cell, thereby holding the antigens in native, immunogenic configuration. In addition, the RNA may function as an intracellular adjuvant. The RNA in our preparations might function similarly, binding an as yet unidentified immunizing component of the mycobacterial cell and maintaining the immunizing configuration. Or, if the immunizing component is of low molecular weight, it may be immunogenic (antigenic?) only when complexed with a large molecule such as RNA. It has been shown that nucleosides can function in this manner (23). In either case, degradation of the RNA by ribonuclease prior to being introduced into cells would greatly reduce the immunizing activity, though not necessarily completely.

Finally, the possibility should be considered that mycobacterial RNA might attach to ribosomes in macrophages, or other cells, and replicate or direct the production of mycobacterial protein in a manner analogous to RNA viruses. This, in turn, might produce a refractory state within these cells, again in a manner analogous to the refractory state produced by latent virus infection. This theory may seem far-fetched, but there are too many unknown aspects of the phenomenon of latency of mycobacteria in healthy hosts to permit it to be arbitrarily dismissed.

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