STUDIES OF TUBERCLE BACILLUS-HISTIOCYTE RELATIONSHIPS

VI. Induction of Cellular Resistance by Ribosomes and Ribosomal RNA*

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The results of investigations, particularly of recent years, have established with some degree of certainty a form of resistance intimately associated with cells.

It has been shown in this context that cellular immunity underlies the rejection of most tissue grafts (1, 2), and it is probable that the resistance of agammaglobulinemics to viral infections is of a similar nature (3). Cellular resistance has also been postulated for a number of microbial diseases, including pasteurellosis (4), brucellosis (5, 6), salmonellosis (7), and tuberculosis (8–11).

While the mode of expression of cell-bound resistance depends upon the nature of the biological phenomenon, the manifestation of resistance in any particular biological event is not necessarily stereotyped. Its expression in microbial infections is certainly of protean nature and includes not only easily demonstrable effects upon the parasite such as the suppression of microbial growth but also more subtle changes in both the parasite and the host; included in the latter category are modification of microbial virulence (12, 13) and induction of resistance in host cells to parasite-induced necrosis (11).

Despite the undisputed existence of cellular resistance and the demonstrable effects of its assertion, it is still not possible to describe in precise terms the fundamental nature of this form of resistance. A few clues are, however, presently available. In the special case of cellular resistance against *Mycobacterium tuberculosis*, where resistance is in part manifested by refractoriness of host cells to necrotization by virulent bacilli, passive transfer of this resistance to normal animals has been demonstrated *via* injections of immune histiocytes derived from animals immunized with the BCG strain of tubercle bacillus. The fact that serial transfer of this cellular resistance was possible and did not require immune histiocytes (*i.e.* histiocytes from animals directly immunized with BCG) and could be achieved by use of histiocytes several passages removed from the original recipients of immune histiocytes suggested a mechanism for continued replication of the cell resistance factor (8). A second observation which afforded insight into the nature of this form of cellular resistance was the demon-

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stration that the histiocytes of normal animals could be rendered resistant by injection of animals with distilled water lysates of immune histiocytes; the induction of resistance in recipients was thus presumably effected by isolated cellular components.

The implications of the above findings have seemed sufficiently important to warrant more intensive studies of this phenomenon. The present paper is largely concerned with identification of the specific cellular component involved in induction of cellular resistance against tubercle bacilli; it also presents data which permit partial chemical characterization of the active component, particularly with respect to its ribonucleic acid nature.

Materials and Methods

Preparation of Cells and Cell Components for Transfer Experiments.—The cell type used in these experiments was the histiocyte derived from the peritoneal cavity of rabbits which had been injected with klearol 5 days earlier. Details concerning the preparation of this cell type and its subcellular components are described below.

(*a*) *Histiocytes:* Since earlier studies (8) have shown that normal histiocytes lacked the capacity for passive transfer of cellular resistance, only rabbits immunized intradermally with the BCG strain of tubercle bacillus were used as histiocyte donors in these experiments.

Details concerning preparation of these cells have been described elsewhere (11); in brief, histiocytes were obtained by washing the peritoneal cavity of each donor rabbit with chilled Tyrode's solution, washing these cells 3 times, resuspending in a small volume of Tyrode's solution, and determining the number of cells in the suspension by counting in a hemocytometer.

Stained preparations of these cell suspensions showed 90 to 95 per cent histiocytes; the remainder consisted largely of polymorphonuclear leucocytes and lymphocytes.

(*b*) *Histiocytic lysates:* Lysates of histiocytes were made by mixing known numbers of washed and packed histiocytes with 5.0 ml of distilled water; the cells were then maintained in a water bath at 37°C for 3 hours with occasional shaking of the sample by hand. At the end of 3 hours the sample was centrifuged for 10 minutes at 250 *g* to remove cellular debris and any unlysed cells; the 5.0 ml of relatively clear supernatant fluid was removed and the entire amount inoculated intradermally into a single rabbit in each transfer experiment.

(*c*) *Cell fractions:* The method of preparation of cell fractions was that described by Hogeboom (14). Briefly, the starting material for the preparation of cell fractions consisted of histiocytes which were suspended in 0.25 M sucrose and homogenized for 4 to 5 minutes with the Potter-Elvehjem homogenizer. Whole histiocytic homogenate was prepared by centrifugation of the starting material for 10 minutes at approximately 750 *g*; the supernatant fluid derived therefrom was used as histiocytic homogenate. The nuclear, mitochondrial, and microsomal fractions were obtained by several cycles of differential centrifugation of the starting material and additional homogenization of certain of the fractions as described by Hogeboom.

The ribosomal fraction was prepared by the method of Littlefield (15). In this procedure, the starting material was layered over 0.34 M sucrose and centrifuged for 15 minutes at approximately 9000 *g*; the supernatant fluid was removed and centrifuged for 1 hour at 105,000 *g*. The sediment obtained after the second centrifugation was resuspended in 0.34 M sucrose, mixed with an equal volume of 5 per cent sodium desoxycholate (dissolved in phosphate-buffered saline, pH 7.2), and centrifuged for 2 hours at 105,000 *g*. The supernatant fluid after centrifugation constituted the solubilized materials whereas the sediment, upon resuspension in 0.25 M sucrose, provided the ribosomal fraction.

(*d*) *Ribosomal RNA:* Ribosomes, prepared as described above, were resuspended in 0.2 M

phosphate buffered saline, pH 7.2 (instead of sucrose). The method of extraction of RNA from ribosomes was essentially that described by Gierer and Schramm (16). An equal volume of water-saturated phenol was added to the ribosomal material and shaken for 8 minutes at 5°C. The water-saline phase was separated from the phenol phase by centrifugation for 8 minutes at 1000 RPM and the former material was reextracted twice, each time with an equal volume of phenol and for 2 minutes at 5°C. After the final extraction, the water-saline phase was treated with ether to remove excess phenol; the ether was in turn removed by bubbling nitrogen through the sample. The material obtained in this manner constituted ribosomal RNA and was used as soon as possible after preparation.

Enzymes.—Ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey, 2 times crystalline) was dissolved in phosphate-buffered saline, pH 7.2; enough enzyme was added to the test material to yield an enzyme concentration of 1.0 mg or 1.0 μg per ml test material.

Deoxyribonuclease (Nutritional Biochemical Corporation, Cleveland) was dissolved in physiological saline which contained 100 mg magnesium sulfate per ml of saline; sufficient enzyme was added to test material to yield 1.0 mg enzyme per ml of sample.

Trypsin (Difco Laboratories, Inc., Detroit) was dissolved in phosphate-buffered saline, pH 7.2, and was used at a final concentration of 1.0 mg enzyme per ml of test material.

The time of exposure of test material to enzyme was 1 hour in the case of the microsomal and ribosomal fractions and only 20 minutes in the case of phenol-extracted ribosomal material. Incubation of these samples was at 37°C.

Induction of Resistance.—Intact immune histiocytes or components derived therefrom were injected intradermally into normal adult rabbits. When large volumes of test material were to be injected, the injections were made into several sites in the skin, each site receiving 0.5 ml of test material. The total numbers of cells or cell equivalents injected varied in different experiments and the values are indicated in the appropriate tables.

Tests for Cellular Resistance.—These tests were made with the peritoneal histiocytes of donor rabbits (BCG-immunized rabbits) and recipient rabbits (rabbits given immune histiocytes or immune histiocytic fractions 13 days previously). The histiocytes were sedimented by centrifugation at 250 *g* for 3 to 4 minutes and redispersed in 6 to 8 ml of freshly prepared 0.25 per cent trypsin in Tyrode's solution. After 30 minutes at 24–26°C, the trypsinized cells were washed 3 times with Tyrode's solution. The packed cells from the last washing were resuspended in a few milliliters of immune serum (from BCG-immunized rabbits) and the numbers of cells present were determined by counting in a hemocytometer. Part of these histiocytes was used as cell controls to show that uninfected cells did not undergo spontaneous degeneration. The rest of the histiocytes was tested for resistance against virulent tubercle bacilli according to the following procedure:

(*a*) *Parasitization of histiocytes:* The H37Rv strain of tubercle bacillus was mixed with the histiocytes in a ratio of approximately 10 bacteria per cell; 0.5 ml of mixture was placed in a paraffin-lined bottle, centrifuged for 10 minutes at 850 *g*, and refrigerated 1 hour at 4°C. After refrigeration the supernatant fluid was discarded and the sedimented cells resuspended in a small volume of immune serum medium. The number of histiocytes was determined in a hemocytometer, and the infected suspension was diluted with additional immune serum medium to yield approximately 15 cells per mm^3; the diluted suspension of infected cells was used for *in vitro* cultures.

(*b*) *Cultivation of histiocytes:* This was carried out in the culture chambers described by Mackaness (17). Approximately 500 to 1000 cells were introduced into the central area of each culture chamber. After adherence of histiocytes to the bottom coverslip, the culture chamber was closed by insertion of the top coverslip. Sufficient immune serum medium was introduced *via* lateral drill holes into the chamber to fill approximately two-thirds of the remaining space within the culture chamber. The chambers were incubated at 37°C.

(c) *Examination of cultures:* The number of histiocytes in the central area of the culture chamber was determined at the start of the experiment and at certain intervals thereafter. Counts were made with a phase contrast microscope and a 10 × objective. The full details concerning enumeration were described previously (11). Cellular resistance was evidenced by absence of cellular degeneration and constancy of cell numbers in infected cell cultures.

Percentage of Infected Histiocytes and Average Number of Bacteria per Infected Cell.—The proportion of infected histiocytes and the numbers of ingested bacteria were determined as follows: a small amount of the parasitized cell suspension was placed on a coverslip; after adherence of the cells to the coverslip, the fluid was removed and the specimen allowed to air-dry; the specimen was fixed with heat, passed through successive changes of xylol and alcohol, and stained by the Ziehl-Neelsen's method.

The percentage of infected histiocytes was determined by counting a total of 200 stained cells. The average number of bacilli per infected cell was obtained by examining 200 stained cells, counting the total number of intracellular bacteria, and dividing this total by the number of infected histiocytes.

Bacteria.—The bacteria used in these studies were the H37Rv and BCG strains of *Mycobacterium tuberculosis.*

The H37Rv strain was grown in tween-albumin liquid medium for 7 days at 37°C. The week-old culture was washed several times in tween-albumin medium; after the last washing the sedimented bacteria were resuspended in a small volume of medium and centrifuged at 250 *g* for 3 minutes to remove larger aggregates. The supernatant fluid obtained in this manner consisted mainly of bacteria occurring singly; after determination of bacterial cell numbers in a Petroff-Hausser chamber under dark ground illumination, the supernatant fluid was used as a source of bacteria for parasitization of histiocytes. The BCG strain of tubercle bacillus used for immunization of rabbits was cultivated on Calmette's potato medium. The bacterial growth from a 2-week-old culture was ground with steel balls, suspended in physiological saline, and diluted to contain 1.0 to 2.0×10^8 bacteria per ml. This bacterial suspension was injected intradermally into rabbits in 0.1 to 0.2 ml amounts.

Nutrient Media.—The media for cultivation of histiocytes consisted of 40 per cent rabbit serum (normal serum or anti-BCG serum) in Tyrode's solution (a modified Tyrode's solution containing no calcium was used). Inclusion of cultures containing normal serum provided the controls for determining that the bacillary culture used for infection of histiocytes was capable of necrotizing cells; however, to keep tables simple, results with cultures in normal serum are not shown in the tables.

The pH of all nutrient media was adjusted to 7.4 with 5 per cent CO_2 in air before use.

Tuberculin Test.—0.2 ml of 1:100 dilution of commercially available old tuberculin was inoculated intradermally into rabbits or guinea pigs; the test was read at 48 hours.

EXPERIMENTAL RESULTS

Stability of Immune Histiocytic Lysates at Various Temperatures.—An earlier report (8) has shown that lysis of immune histiocytes (derived from BCG-immunized rabbits) in distilled water yielded a cell lysate which, when inoculated intradermally into normal recipient animals, rendered the histiocytes of these recipients refractory to the necrotizing action of virulent tubercle bacilli. In order to identify and to characterize the active component of these immune cell lysates, prior knowledge of the stability of these immune lysates was needed; immune lysates from resistant histiocytes were therefore prepared and exposed for varying periods of time to several different temperatures and

subsequently tested for their ability to induce cellular resistance in normal animals. The results of one of a series of similar experiments are presented in Table I.

TABLE I

Stability of Immune Histiocytic Lysate at Various Temperatures

Treatment of immune lysate*	No. cell equivalent (× 10^8) inoculated into recipient‡	Infected histiocytes§	Average No. bacilli per histiocyte§	Average degeneration,‖ hrs. after infection	
				24	48
		per cent		*per cent*	*per cent*
Untreated	22.0	16	3	0	0
Incubated 24 hrs. at 25°C	22.0	14	2	0	0
Untreated	4.5	13	3	0	1
Incubated 24 hrs. at 37°C	4.5	17	3	0	0
Untreated	3.2	20	3	0	0
Incubated ½ hr. at 56°C	3.2	16	3	24	52
Incubated ½ hr. at 56°C	3.2	0	0	0	0

* Lysates were prepared by adding distilled water to immune histiocytes (derived from BCG-immunized rabbits) and incubating the mixture for 3 hours at 37°C; after incubation, the sample was centrifuged to sediment cellular debris and the supernatant fluid used as immune lysate.

‡ Figures represent numbers of histiocytes which would have been inoculated into recipients if cells had not been lysed. The immune lysates were inoculated intradermally into normal adult rabbits.

§ Values indicate per cent of infected recipient histiocytes and average numbers of bacilli per infected histiocyte immediately after infection of cells with virulent bacilli. The histiocytes of recipient rabbits were tested for cellular resistance 13 days after intradermal inoculation of lysate.

‖ Represents average per cent degeneration (from initial count of approximately 500 to 1000 histiocytes per culture chamber) in 2 to 3 replicate cultures at 24 and 48 hours after infection. All the necessary uninfected cell controls in serum media were included in each experiment, but generally only the results obtained with the infected experimental samples are shown in detail in this and subsequent tables; the results obtained with uninfected control cultures are included only when needed to demonstrate a point.

The ability of lysates to initiate development of cellular resistance in normal recipients is indicated by the absence of cellular degeneration in *in vitro* cultures of recipient histiocytes after infection of these cells with the virulent H37Rv strain of tubercle bacillus. Table I indicates quite clearly that the active component of immune histiocytic lysates was stable for at least 24 hours at 25–37°C (no degeneration of infected histiocytes at either 24 or 48 hours after infection) but was inactivated after exposure for 30 minutes at 56°C (24 to 52

per cent degeneration of cells). The cell degeneration noted in these latter cultures was not due to any inherent instability of histiocytes from animals inoculated with heated lysate, for the uninfected histiocytes of these animals were readily maintained in *in vitro* cultures (last row of table). While there was some variation in percentage infection of histiocytes and in average numbers of bacilli per infected histiocyte, it is quite evident that the slight variations among the different experimental samples could not account for the observed marked differences in cellular degeneration.

Effect of Nucleases upon Immune Histiocytic Lysates.—Earlier studies of passive transfer of cellular resistance by immune histiocytes had shown that serial passive transfer of resistance was possible, thus suggesting some sort of mechanism for production of the cell resistance factor. In the light of modern biological thinking, it seemed likely that one or the other of the two major classes of nucleic acid was involved in replication of the factor or factors concerned with cellular resistance. Immune histiocytic lysates were therefore prepared and subjected to the action of either ribonuclease or deoxyribonuclease prior to testing of their ability to effect transfer of cellular resistance. The results of these investigations are shown in Table II.

Since it was deemed desirable to clarify immune histiocytic lysates as much as possible prior to exposure of lysate to enzyme, the histiocytic lysates were centrifuged for 45 minutes at an arbitrarily chosen speed of 11,500 RPM. As shown in the upper half of Table II, the component responsible for transfer of cellular resistance was entirely confined to the supernatant fluid after centrifugation; thus, histiocytes derived from animals inoculated with supernatant fluid withstood necrotization by virulent tubercle bacilli whereas histiocytes of animals inoculated with the sediment of centrifuged lysates showed 22 and 54 per cent degeneration at 24 and 48 hours post infection.

The data in the lower part of Table II reveal that intradermal injection of the untreated supernatant fluid of centrifuged immune lysates or of deoxyribonuclease-treated supernate into normal recipient animals resulted in induction of cellular resistance (no degeneration of recipients' histiocytes after infection with virulent H37Rv). Injection of ribonuclease-treated supernate, on the other hand, failed to induce cellular resistance in recipient animals and 27 to 53 per cent of their histiocytes had undergone degeneration after 24 to 48 hours of infection by virulent bacilli. Inasmuch as the histiocytes of animals given ribonuclease-treated fluid were not intrinsically unstable (as evidenced by the last row of Table II which shows unimpaired capacity of these cells to survive when uninfected), the failure of ribonuclease-treated material to induce cellular resistance implied an important role for ribonucleic acid in this phenomenon.

Activity of Various Cell Fractions in Induction of Cellular Resistance.—The occurrence of ribonucleic acid in nuclear as well as cytoplasmic regions of the cell is well known, and the suggested importance of ribonucleic acid in transfer

of cellular resistance emphasized the need for analyses of cell fractions for their ability to induce cellular resistance in normal animals. Accordingly, preparations of immune histiocytes were fractionated by the method of Hogeboom (14) and individual fractions (nuclear, mitochondrial, microsomal, and ribosomal) were tested for their ability to initiate development of cellular resistance in normal recipient animals. The results of these investigations are presented in Table III.

TABLE II

Effect of Nucleases upon Immune Histiocytic Lysates

Treatment of immune lysate	No. cell equivalent (× 10^9) inoculated into recipient	Infected histiocytes	Average No. bacilli per histiocyte	Average degeneration, hrs. after infection	
				24	48
		per cent		*per cent*	*per cent*
Untreated*	8.0	16	2	0	0
Centrifugation (supernate)*	8.0	17	3	0	0
Centrifugation (sediment)*	8.0	16	3	22	54
Centrifugation (supernate)‡	46.2	15	3	0	0
Centrifugation and RNAase‡	46.2	17	3	27	53
Centrifugation and DNAase‡	46.2	15	3	0	1
Centrifugation and RNAase‡	46.2	0	0	0	1

See footnotes in Table I.

* Immune histiocytic lysate was prepared in manner described under Materials and Methods. Part of this lysate was centrifuged for 45 minutes at an arbitrarily selected speed of 11,500 RPM; the supernatant fluid and the sedimented material were separated and used for intradermal inoculation of normal rabbits. As control, the untreated lysate was inoculated into a recipient rabbit.

‡ An immune lysate was prepared, clarified by centrifugation at 11,500 RPM for 45 minutes and the supernatant fluid used as a source of active material for passive transfer of cellular resistance. Part of this material was treated with ribonuclease (1 mg RNAase/ml supernatant) or with deoxyribonuclease (1 mg DNAase/ml supernatant) prior to inoculation into recipient rabbits.

Since the method of preparing cell fractions required sucrose as diluent, it was necessary to show that sucrose did not interfere with the transfer of cellular resistance; for this purpose, a homogenate of immune histiocytes was prepared in sucrose and tested for its ability to induce cellular resistance. As shown in the first two rows of the table, intradermal injection of a histiocytic homogenate resulted in development of cellular resistance similar to that occasioned by injection of intact immune histiocytes (no evidence of histiocytic degeneration after infection with virulent bacilli in either sample).

Similar tests with nuclear, mitochondrial and microsomal fractions prepared from immune histiocytes revealed that the nuclear and mitochondrial fractions

were inactive (histiocytes of recipients given these fractions exhibited degeneration ranging from 21 to 48 per cent during the first 48 hours after infection). The histiocytes of animals given microsomal fraction, however, proved

TABLE III

Activity of Various Cell Fractions in Passive Transfer of Cell Resistance

Substance used in passive transfer	No. cell equivalent (× 10^8) inoculated into recipient*	Infection of histiocytes	Average No. bacilli per histiocyte	Average degeneration, hrs. after infection	
				24	48
		per cent		*per cent*	*per cent*
Immune histiocytes‡	8.5	13	4	0	1
Histiocytic homogenate‡	34.0	13	2	0	0
Nuclear fraction‡	34.0	17	3	21	47
Mitochondrial fraction‡	34.0	14	3	26	48
Microsomal fraction‡	34.0	16	3	0	0
Microsomal fraction§	127.0	17	3	0	0
Soluble fraction§	127.0	21	4	33	55
Ribosomal fraction§	127.0	21	4	0	0
Soluble fraction§	127.0	0	0	0	2
Ribosomal fraction‖	25.6	21	4	0	0
Ribosomal-RNAase‖	25.6	17	4	27	53
Ribosomal-RNAase‖	25.6	0	0	0	0

See footnotes in Table I.

* Represents the numbers of histiocytes which yielded the amount of nuclear, mitochondrial, microsomal, or ribosomal material inoculated into individual recipient animals.

‡ A concentrated suspension of immune histiocytes was divided into several parts and these were used to prepare the cell fractions; the histiocytic lysate as well as the various cell fractions contained sucrose as diluent.

§ A microsomal fraction prepared from immune histiocytes was divided into two parts. One part was untreated and served as indicator of the activity of the microsomal fraction (*i.e.*, ability to effect passive transfer of resistance). The rest of the microsomal fraction was treated with 5 per cent sodium desoxycholate; after centrifugation of the desoxycholate-treated material, the supernatant fluid and the sediment were separated and used as soluble fraction and ribosomal fraction respectively.

‖ A ribosomal fraction was prepared from a suspension of immune histiocytes and part of this ribosomal fraction was exposed to ribonuclease (1.0 mg RNAase/ml of fraction) before injection into recipient rabbits.

completely resistant, for no degeneration of infected histiocytes was evident after 48 hours of infection. Since neither the percentage of infected histiocytes nor the average numbers of bacilli per infected histiocyte is significantly different in these experiments, it is clear that there is a major difference in the activity of the various cell fractions.

Further identification of the active material was made by treatment of an immune microsomal fraction with sodium desoxycholate. Treatment of the microsomal fraction with this chemical resulted in solubilization of the membranes so that upon centrifugation of the desoxycholate-treated material, a supernatant fluid containing the solubilized materials and a sediment composed of granular ribosomal substance were obtained. Tests of the activity of these two substances (rows 7 and 8 of Table III) indicated that the ribosomal fraction but not the soluble fraction was able to render the histiocytes of normal recipients resistant to necrotization by virulent tubercle bacilli. It is also apparent that the histiocytes of animals inoculated with soluble fraction are not inherently unstable (uninfected histiocytes of row 9 show no degeneration).

In view of the presumed importance of ribonucleic acid in induction of cellular resistance, as suggested by the abolition of the activity of histiocytic lysates through exposure of lysate to ribonuclease, it seemed of importance to determine whether the activity of the ribosomal fraction was also susceptible to inactivation by ribonuclease. As shown in the lower part of Table III, the activity of an immune ribosomal fraction was indeed destroyed by exposure to the enzyme, for the histiocytes of animals given ribonuclease-treated ribosomes failed to resist necrotization by virulent tubercle bacilli; the 27 to 53 per cent degeneration of these histiocytes at 24 and 48 hours post-infection was definitely not attributable to instability of histiocytes caused by injection of enzyme-treated material, since the uninfected histiocytes of these same animals (last row of table) showed no evidence of degeneration.

The above observations are thus indicative of a major association of the cell resistance–inducing factor with the ribosomal fraction of the cell and more specifically with the ribonucleic acid of this cell fraction. While there may be reservations concerning the purity of subcellular fractions prepared according to currently available techniques, it seems highly unlikely that the activity of the ribosomal fraction was due to contamination of this fraction by other subcellular units since none of the other components proved active.

Activity of Ribosomal RNA in Induction of Cellular Resistance.—The importance of ribonucleic acid in induction of cellular resistance, as suggested by the preceding findings, was further studied by extraction of immune ribosomes with phenol. The ribosomal RNA obtained in this manner was tested for its ability to induce cellular resistance in normal animals. Representative results from two of a number of similar experiments are shown in Table IV.

Examination of the upper part of Table IV reveals that animals which had been injected intradermally 13 days earlier with ribosomal RNA yielded histiocytes which were resistant to virulent tubercle bacilli. This is evident when the results in the first two rows are compared; in the presence of immune (anti-BCG) rabbit serum which is needed for mediation of cellular resistance (11), there was an early degeneration of infected histiocytes; however, no further

loss of cells occurred after 24 hours. In contrast, infected histiocytes maintained in normal rabbit serum showed progressive degeneration of cells so that at 72 hours postinfection, 71 per cent of infected histiocytes had been destroyed.

TABLE IV

Activity of Ribosomal RNA in Induction of Cellular Resistance

Substance used in passive transfer*	No. cell equivalent ($\times 10^9$) inoculated into recipient	Infection of recipient histiocytes	Average No. bacilli per infected histiocyte	Serum for cultivation of infected histiocytes‡	Average degeneration, hrs. after infection		
					24	48	72
		per cent			*per cent*	*per cent*	*per cent*
Untreated RNA	6.0	21	4	NRS	37	53	71
Untreated RNA	6.0	23	3	IRS	24	22	22
RNAase-treated RNA	6.0	21	4	NRS	32	50	68
RNAase-treated RNA	6.0	22	4	IRS	17	58	71
DNAase-treated RNA	6.0	19	4	NRS	26	43	58
DNAase-treated RNA	6.0	19	4	IRS	23	24	24
Trypsin-treated RNA	6.0	21	3	NRS	19	53	70
Trypsin-treated RNA	6.0	22	4	IRS	27	31	36
RNA kept at 37°C	6.0	21	4	NRS	34	50	66
RNA kept at 37°C	6.0	22	4	IRS	22	22	22
Untreated RNA	3.5	23	4	NRS	20	49	—
Untreated RNA	3.5	24	4	IRS	6	6	—
RNAase-treated RNA	3.5	20	3	NRS	20	57	—
RNAase-treated RNA	3.5	19	4	IRS	35	62	—

All uninfected cell cultures behaved normally (i.e., no degeneration); results not shown in table.

All of the above enzymes were tested against appropriate substrates (yeast RNA, Nutritional Biochemical Co.; calf thymus DNA, Worthington Biochemical Corporation; and casein) and found to be active.

—, indicates sample not tested.

See footnotes in Table I.

* Untreated RNA was kept at 4°C for 20 minutes; all other samples of RNA were kept at 37°C for 20 minutes. RNAase and DNAase designate ribonuclease and deoxyribonuclease respectively. Concentration of enzymes used: upper part of table, 1.0 mg per ml sample; lower part, 1.0 μg per ml.

‡ NRS designates normal rabbit serum medium; IRS indicates medium containing immune serum from BCG-immunized rabbits.

Upon comparison of these results with those shown in Table III, it would seem that induction of cellular resistance is less efficient with ribosomal RNA than with intact ribosomes. Inasmuch as the uninfected histiocytes of animals given untreated ribosomal RNA were not intrinsically unstable in *in vitro* cultures (results not shown in table), it would appear that the initial degenera-

tion of infected histiocytes represented destruction of non-resistant cells. This apparent failure of ribosomal RNA to convert an entire population of histiocytes to resistance against virulent tubercle bacilli may be explained in various ways. One interpretation is that the uptake of ribosomes by histiocytes is more efficient than that of ribosomal RNA so that conversion of cells to resistance is proportionally greater with ribosomes; the present situation may therefore be analogous to that of infectious viral RNA, for infection of susceptible cells by viral RNA is highly inefficient as compared to intact virus. On the other hand, it is just as likely that incomplete conversion of the histiocytic population was a reflection of the low activity of the ribosomal RNA used in this experiment; as shown in the lower part of Table IV (rows 11 and 12) it is possible to obtain a preparation of ribosomal RNA capable of converting almost an entire population of histiocytes to resistance (the 6 per cent degeneration in row 12 is just barely over the allowance of 5 per cent error in counting of cells).

Exposure of ribosomal RNA to ribonuclease resulted in marked loss of activity; thus, upon infection of the histiocytes of animals inoculated with ribonuclease-treated material, there was progressive degeneration of cells in cultures maintained in either normal or immune serum medium (rows 3 and 4 show results after use of 1.0 mg RNAase per ml sample; in rows 13 and 14, only 1.0 μg of RNAase per ml sample was used). The histiocytes of animals given deoxyribonuclease-treated ribosomal RNA (1.0 mg DNAase per ml sample) proved resistant when infected histiocytes were maintained in immune serum medium but not in normal serum medium (rows 5 and 6). Treatment of ribosomal RNA with trypsin (1.0 mg enzyme per ml sample) caused only a minor loss of activity; as shown in row 8, infection of the histiocytes of animals given trypsin-treated ribosomal RNA resulted in a slightly greater cellular degeneration than that noted for the histiocytes of animals receiving untreated or deoxyribonuclease-treated material (*i.e.*, 36 per cent degeneration in row 8 as against 22 to 24 per cent in rows 2 and 6 after 72 hours of infection). It is however quite clear that the action of trypsin upon ribosomal RNA is not comparable to that of ribonuclease (row 4).

The data in row 10 show that incubation of ribosomal RNA for 20 minutes at 37°C did not result in any loss of activity; it is therefore apparent that the marked reduction in activity after treatment of ribosomal RNA with ribonuclease was attributable to enzyme action and not to heat inactivation.

Tests for Presence of Viable Bacilli in Ribosomal Fraction.—Although the contention that viable bacilli may be transferred in these experiments and may be responsible for induction of cellular resistance appears to be obviated by the demonstrated capacity of ribosomal RNA to induce cellular resistance (the killing of tubercle bacilli and the denaturation of proteins by phenol are established facts), tests for presence of viable bacilli in the ribosomal fraction were made nonetheless. For this purpose, two guinea pigs were injected subcu-

taneously in the region of the groin with amounts of ribosomal material (not treated with phenol) equivalent to 4.0×10^9 histiocytes. These animals were entirely negative when tuberculin tested at 4 and 8 weeks after injection of ribosomes. Inasmuch as the relatively susceptible guinea pig failed to show signs of infection when injected with very large amounts of ribosomal material, it seems highly improbable that induction of cellular resistance in the more resistant rabbit resulted from infection of these animals with residual viable bacilli. The sera of these ribosome-treated guinea pigs, when tested for presence of complement-fixing antibody using whole bacilli and mechanically disrupted BCG as antigens, also yielded negative results. Although only two guinea pigs were used in these tests, it is quite clear that this is of less importance than the quantity of ribosomal material injected; in this respect, it should be noted that the amount of ribosomal material inoculated into guinea pigs represented an amount which consistently proved capable of inducing cellular resistance in recipient rabbits. It is also to be emphasized that the results reported in this section constitute only part of the body of evidence against the participation of residual viable bacilli or bacillary antigens in induction of cellular resistance. As presented in the discussion section of this paper, there are additional and more cogent reasons for acceptance of the role of cellular components in establishment of cellular resistance.

DISCUSSION

Use of the term cellular resistance is sanctioned by the repeated demonstration of cell-mediated resistance in such diverse phenomena as homograft immunity and resistance against certain viral and bacterial agents. While this term is a useful and descriptive one and permits categorization of one form of resistance, its true significance can be grasped only when precise knowledge of the mechanisms involved is available. The mechanistic investigation of cellular resistance may be approached in a number of ways; the one which has been used in this work has sought to identify the particular cell component involved in induction of cellular resistance and to determine some of its physical-chemical characteristics.

These investigations have shown that a substance capable of inducing cellular resistance was present in the microsomal fraction of the cell and more specifically that it was associated with the ribosomal material. The importance of ribonucleic acid in induction of cellular resistance was first suggested by the inactivation of ribosomes after exposure to ribonuclease; further evidence of its importance was indicated by the ability of isolated ribosomal RNA to initiate development of cellular resistance in normal animals and by the abolition of this activity by treatment with ribonuclease but not deoxyribonuclease or trypsin.

The results reported herein may be interpreted in several ways. It might be

postulated that the histiocytes were latently infected with an RNA virus and that activation of virus occurred after immunization of animals with tubercle bacilli; upon implantation of activated virus in recipient animals, infection of histiocytes occurred with consequent modification of cellular metabolism and development of resistance to virulent tubercle bacilli (*e.g.*, activation of cellular enzymes by viral infection may inhibit formation of bacterial components of virulence). A second interpretation is that immunization of animals with bacilli resulted in more or less permanent modification of host DNA (the prolonged residence of tubercle bacilli within host cells certainly provides ample time for possible mutagenic effects); the modification of host DNA could then lead to formation of altered RNA and thence to production of modified proteins (enzymes) which inhibit formation of bacterial virulence factors. While this interpretation may not be entirely consistent with present concepts of messenger RNA, particularly in respect to the apparent stability of our ribosomal RNA, it must be emphasized that the generalization concerning the lability of messenger RNA is based on extremely limited observations as far as mammalian cells are concerned. A third interpretation might be that immunization of animals resulted in activation of a mechanism which originally derives its information from the genetic material but which is capable of self-replication after activation. This possibility has been advanced by Fox *et al.* (18) as one explanation of their findings of a structural modification of a specific protein in *Drosophila melanogaster* in the absence of the genetic material responsible for the formation of this protein; these workers suggested that the pertinent mechanism might consist of an enzymatic system for the synthesis of RNA using preformed RNA as "primer." Various aspects of these three interpretations are experimentally verifiable and preliminary studies of these hypotheses are under way.

The biological activity of ribonucleoproteins and ribonucleic acids has been studied by various other investigators. It has been reported that these substances may effect functional and morphological modifications in vertebrate systems and alterations in homograft tolerance and antibody formation (19–22). It would seem of interest to determine whether the active material in these instances was associated with ribosomes or some other cellular component.

The uptake of ribosomes or ribosomal RNA by host cells, presumably histiocytes, is probably a requirement for induction of cellular resistance. While the present work has not concerned itself with cellular uptake of active materials, there are many recent reports which indicate that labeled RNA can be taken in by cells both *in vitro* and *in vivo* (20, 23, 24).

Acceptance of the concept of induction of cellular resistance by host ribosomal RNA obviously depends upon proof that viable bacilli or bacillary antigens are not involved in induction. Some evidence of this has been presented in an earlier report (8) and additional proof may be extracted from this paper. The nature of this evidence may be summarized as follows: (*a*) absence of

acid-fast bacilli in stained preparations of immune rabbit histiocytes, (*b*) absence of tuberculin reactivity and complement-fixing antibodies in guinea pigs inoculated with large quantities of immune ribosomes, and (*c*) induction of cellular resistance by ribosomal RNA obtained from material extracted with phenol, a known bactericidal and protein-denaturing agent. Elimination of bacillary antigens as inducing agent is additionally based upon successful serial transfer of resistance with the inevitable elimination of nonreplicating materials. However, the observations which are most incompatible with the contention that induction of cellular resistance in these studies was due to transfer of bacilli and bacillary antigens are those pertaining to interspecies transfer of cellular resistance (manuscript in preparation). In these studies of interspecies transfer of resistance it was found that injection of 6.7×10^8 immune rabbit histiocytes (from BCG-immunized rabbits) into normal guinea pigs rendered the recipients' histiocytes resistant to virulent tubercle bacilli; in contrast, injection of 2.1×10^9 immune guinea pig histiocytes (from BCG-immunized guinea pigs) failed to induce resistance in normal guinea pigs. To explain these results in terms of transfer of bacilli and bacillary antigens, the assumption must be made that immune rabbit histiocytes transferred an adequate amount of bacillary material to cause active induction of cellular resistance whereas 3 times as many immune guinea pig histiocytes failed to do so. The implausibility of this assumption becomes even greater when consideration is given to the inherently greater resistance of rabbits to infection with tubercle bacilli and to the relative chances of transfer of bacilli by immune rabbit but not by immune guinea pig histiocytes in the light of this difference in resistance of the two animal species.

SUMMARY

The various cellular components of immune rabbit histiocytes have been analyzed for their ability to induce cellular resistance in normal animals. The results of these investigations have shown that the nuclear and mitochondrial fractions were inactive and that the microsomal and ribosomal fractions were active.

The importance of ribonucleic acid in induction of cellular resistance was established by isolation of an active ribosomal RNA and by demonstration of inactivation of this material with ribonuclease but not with deoxyribonuclease or trypsin.

The possibility that viable bacilli were present in immune ribosomes was tested; the absence of complement-fixing antibodies and of skin reactivity to tuberculin in animals inoculated with ribosomes was considered as partial evidence of absence of living bacilli.

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