STUDIES OF TUBERCLE BACILLUS–HISTIOCYTE RELATIONSHIPS*

VII. Homologous and Heterologous Transfer of Cellular Resistance

By JACOB FONG, Ph.D., DENNIS CHIN, and HERTA M. VICKREY‡

(From the Department of Bacteriology, University of California, Berkeley)

(Received for publication, July 22, 1963)

A form of cellular resistance which has been intensively investigated in our laboratories is one that is directed against *Mycobacterium tuberculosis*. This cellular resistance, which is readily demonstrated with the histiocytes of rabbits immunized with the BCG strain of tubercle bacillus, is expressed in a number of ways depending upon the conditions of experimentation; one expression of this resistance is refractoriness of immune histiocytes to the necrotizing action of virulent bacilli (1).

More recent studies of this type of cellular resistance have shown that the capacity to resist necrotization by virulent tubercle bacilli could be passively transferred to homologous species (rabbit to rabbit) *via* injections of not only immune histiocytes but also distilled water lysates of such histiocytes (2); investigations aimed at more precise characterization of the subcellular component of immune histiocytes responsible for induction of cellular resistance in recipient animals indicated that the activity resided in the ribosomal fraction of the histiocyte and more specifically in the ribosomal ribonucleic acid (3).

There are at least two pertinent questions which arise in connection with these studies: (*a*) Are these findings of limited applicability which pertain only to the rabbit with its relatively high resistance to the human strain of tubercle bacillus, or do these findings constitute basic phenomena applicable to other inherently more susceptible animal species as well? (*b*) Is interspecies transfer of cellular resistance against mycobacteria possible in view of the seemingly rather fundamental nature of the active component responsible for induction of cellular resistance?

The present paper reports the results of investigations specifically designed to answer these two questions. It describes induction of cellular resistance in rabbits, guinea pigs, and mice by immunization with BCG or by intradermal injections of immune histiocytes; it also reports on successful interspecies

* This work was supported by a grant from the United States Public Health Service (Grant No. E-1372).

‡ A part of this work was submitted by Herta M. Vickrey to the Graduate Division, University of California, in partial fulfillment of requirements for the M.A. degree.

transfer of cellular resistance with immune histiocytes or histiocytic ribosomes, the reciprocity of transfer between certain of the animal species, and the peculiar behavior of guinea pigs in this respect.

Materials and Methods

Detailed descriptions of procedures and materials not previously used are presented below; those which were employed in earlier studies (1–3) are briefly described herein.

Rabbits.—Adult male rabbits were used in all the experiments reported herein; these rabbits served as donors of normal or immune serum and cells, and as recipients in transfer experiments.

Immune donors consisted of rabbits immunized with the BCG strain of tubercle bacillus; 2.5×10^8 bacilli were inoculated intradermally; only tuberculin-positive animals were used.

Rabbit histiocytes were obtained by washing the peritoneal cavity of each donor rabbit with 200 ml of chilled Tyrode's solution 5 days after intraperitoneal injection of 50 ml of klearol. The suspension of cells was centrifuged at 250 *g* for 3 to 4 minutes, and the sedimented cells were washed 3 times with 150 to 200 ml of fresh Tyrode's solution at each washing. After the final washing, the histiocytes were suspended in a small volume of Tyrode's solution, and the numbers of cells present were determined by counting in a hemocytometer. Cells prepared in this way were used in transfer experiments within 5 hours of harvesting; in tests for cellular resistance, additional treatment of histiocytes was needed, as described below.

Histiocytic lysate was made by lysing known numbers of cells in 5.0 ml of distilled water as previously described (2).

Immune ribosomes were prepared according to the procedure of Littlefield (4). The details of this procedure, as applied to histiocytes, have been previously described (3).

Mice.—The mice for these *in vitro* studies of cellular resistance were 4- to 5-week-old males of the Swiss strain of albino mice. These mice were used as serum and cell donors, and as recipients in transfer experiments. Donors of immune serum and cells were immunized by intradermal injections of approximately 5.0×10^7 viable tubercle bacilli (BCG); these animals were not used until 30 or more days later. The histiocytes were obtained from mice in a manner similar to that employed for rabbits except that a smaller volume of klearol (0.75 ml) was injected for elicitation of histiocytes and a smaller volume of Tyrode's solution was utilized for washing out the peritoneal cavity of each mouse (peritoneum was washed 3 times using 4.0 ml of solution each time). The preparation of these histiocytes for tests of cellular resistance is described below; their preparation for use in passive transfer experiments followed the procedures employed for rabbit histiocytes.

Guinea Pigs.—Male guinea pigs of 600 to 750 gm weight were employed as serum donors, cell donors, and cell recipients in these studies. Immunization of guinea pigs was by intradermal injection of 1.0×10^8 viable bacilli (BCG); only tuberculin-positive animals were used as immune donors. The collection and preparation of guinea pig histiocytes resembled that of rabbits except that 10 ml of klearol was injected for elicitation of histiocytes, and 20 ml of Tyrode's solution was used for washing of the peritoneal cavity.

Passive Transfer Procedure.—Intact histiocytes, histiocytic lysates, or ribosomes were injected intradermally into normal recipient animals. The injections were made into several sites in the skin; the number of sites injected depended on the total volume of material to be injected. The total number 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 histiocytes of donor animals (normal and BCG-immunized rabbits, guinea pigs, and mice) and recipient animals (animals given normal or immune donor cells, or their subcellular fractions 13 days earlier). Details concerning the test for cellular resistance have been previously described (2). Briefly, the

procedure was as follows: part of the histiocytes which had been collected from donor animals or recipient animals was sedimented by centrifugation and redispersed in a small volume of 0.25 per cent trypsin in Tyrode's solution. After 30 minutes of trypsinization the cells were washed and sedimented, and resuspended in a few milliliters of homologous normal or immune serum. The number of cells present was determined by counting in a hemocytometer. Some of these histiocytes were used as cell controls to determine that the uninfected cells used in the experiments did not undergo spontaneous degeneration. The remaining histiocytes were tested for resistance against virulent tubercle bacilli; this test consisted of mixing the H37Rv strain of tubercle bacillus with histiocytes in a ratio of approximately 10 bacteria per cell; 0.5 ml of the 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 homologous normal or immune serum medium. The number of histiocytes was determined in a hemocytometer, and the infected suspension was diluted with additional normal or immune serum medium to yield approximately 15 cells per mm^3; the diluted suspension of infected cells was used for *in vitro* cultures as described below.

Cultivation of Histiocytes.—This was carried out in the culture chambers described by Mackaness (5). Approximately 500 to 1000 uninfected or infected histiocytes were introduced into the space delineated by a plastic ring affixed to the bottom coverslip of the culture chamber. After adherence of histiocytes to the bottom coverslip, the culture chamber was closed by insertion of the top coverslip. Sufficient normal or immune serum medium was introduced *via* lateral drill holes in the chamber to fill approximately two-thirds of the remaining space within the culture chamber. The chambers were incubated at 37°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 (1). Cellular resistance was evidenced by absence of cellular degeneration and constancy of cell numbers in infected cell cultures.

Percentage of Infected Histiocytes.—This was determined by counting a total of 200 stained cells. The method of preparing stained specimens has been described earlier (1).

Average Number of Bacteria per Infected Histiocyte.—This 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*.

For use in parasitization of histiocytes, 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 was found to consist mainly of bacteria occurring singly; after determination of bacterial cell numbers in a Petroff-Hausser chamber under darkground illumination, the supernatant fluid was used as a source of bacteria in parasitization of histiocytes.

The BCG strain of tubercle bacillus was cultivated on Calmette's potato medium. For immunization of animals the bacterial growth from a 2-week-old culture was ground with steel balls, suspended in physiological saline, and diluted to contain the desired number of bacteria per milliliter.

Nutrient Media.—The media used for cultivation of histiocytes consisted of 40 per cent homologous serum (normal serum or anti-BCG serum) in Tyrode's solution (a modified Tyrode's solution containing no calcium was used). The pH of all nutrient media was adjusted to 7.4 with 5 per cent CO_2 in air before use.

EXPERIMENTAL RESULTS

Resistance of Guinea Pig and Mouse Histiocytes to Virulent Bacilli.—Earlier investigations (1) have shown that the histiocytes of rabbits immunized with the BCG strain of tubercle bacillus resisted necrotization by virulent tubercle bacilli and that expression of this resistance required the presence of immune serum; these studies also demonstrated that this type of cellular resistance was passively transferable to normal rabbits by injections of either immune histiocytes or cellular components derived from such histiocytes (2, 3). Since the rabbit is highly resistant to the H37Rv strain of tubercle bacillus used in testing cellular resistance, the general applicability of these observations to the histiocytes of more susceptible animals may be questioned. The present investigations have examined this problem, and the results are presented in this and the next section.

Studies of cellular resistance with the histiocytes of normal and BCG-immunized guinea pigs are shown in the upper half of Table I. The results indicated that normal guinea pig histiocytes were devoid of resistance (note extensive degeneration of infected cells in rows 5 and 6); the degeneration of these cells was not attributable to instability of guinea pig cells in tissue culture, for the uninfected histiocytes of these animals did not undergo degeneration (rows 1 and 2). The degeneration of immune guinea pig histiocytes infected with virulent bacilli and cultured in normal serum media (rows 7 and 9) resembled that of infected normal histiocytes. Infected immune guinea pig histiocytes cultured in immune serum, on the other hand, proved resistant; this was evidenced either by complete absence of degeneration (row 8) or by an initial minor loss which did not progress with time (row 10).

The data in the lower half of Table I show the results of tests of cellular resistance with the histiocytes of normal mice and BCG-immunized mice. It is apparent that infection of normal mouse histiocytes resulted in marked degeneration of infected cells and that this degeneration occurred in the presence of either normal or immune serum medium (rows 15 and 16). The degeneration of immune mouse histiocytes after infection with the H37Rv strain of tubercle bacillus showed, however, a definite relationship to the type of serum present in the culture medium; thus, there was a great loss of infected cells in normal serum medium (row 17) but no loss of cells in immune (from BCG-immunized mice) serum medium (row 18). The percentages of infection of histiocytes in this set of experiments were sufficiently similar and therefore not directly responsible for the observed differences in cell degeneration. Since uninfected normal and immune mouse histiocytes showed no tendency toward spontaneous degeneration (rows 11 to 14), the observed difference between normal and immune histiocyte apparently reflected their different levels of resistance to virulent bacilli.

The above findings indicate, therefore, that mouse histiocytes resembled

TABLE I

Resistance of Guinea Pig and Mouse Histiocytes to Virulent Bacilli

Type of histiocyte tested*	Infected histiocytes‡	Serum medium for culturing histiocytes§	Average degeneration‖	
			24 hr. after infection	48 hr. after infection
	per cent		*per cent*	*per cent*
1. Normal GP.....	0	Normal serum	0	1
2. Normal GP.....	0	Immune serum	0	0
3. Immune GP.....	0	Normal serum	0	0
4. Immune GP.....	0	Immune serum	0	0
5. Normal GP.....	14	Normal serum	32	49
6. Normal GP.....	10	Immune serum	27	53
7. Immune GP.....	14	Normal serum	37	62
8. Immune GP.....	13	Immune serum	2	3
9. Immune GP.....	13	Normal serum	31	59
10. Immune GP.....	13	Immune serum	13	10
11. Normal M.....	0	Normal serum	0	0
12. Normal M.....	0	Immune serum	0	0
13. Immune M.....	0	Normal serum	1	2
14. Immune M.....	0	Immune serum	0	0
15. Normal M.....	17	Normal serum	19	52
16. Normal M.....	15	Immune strum	20	48
17. Immune M.....	15	Normal serum	22	51
18. Immune M.....	22	Immune serum	2	0

* GP and M designate guinea pig and mouse respectively; immune GP and M histiocytes were derived from animals immunized intradermally 30 or more days earlier with approximately 1×10^8 and 5.0×10^7 bacilli (BCG) respectively.

‡ Histiocytes shown in column 1 were divided into 2 parts; one part was not infected with bacilli and was used as normal cell control to detect spontaneous cell degeneration; the second part was infected *in vitro* with H37Rv and the percentage of infected cells was determined immediately after infection. The average number of bacilli per infected cell is not shown in this and subsequent tables since the averages consistently ranged between 3 and 5 in different samples.

§ Samples of uninfected or infected histiocytes were placed into culture chambers and medium consisting of 40 per cent serum in Tyrode's solution was added; serum consisted of homologous normal or immune (BCG-immunized animals) serum.

‖ Represents average per cent degeneration (from initial count of approximately 500 to 1000 histiocytes introduced into each culture chamber) in 2 or more replicate cultures. Cell degeneration of 5 per cent or less is not considered as significant since this is within the limits of error in counting of cells.

rabbit histiocytes in their inducibility to resistance following immunization of animals with BCG and their dependence upon the presence of immune serum for full expression of resistance. While immunization of guinea pigs did not always result in total conversion of the histiocyte population to resistance

against virulent bacilli, the data clearly show that the majority of histiocytes from immunized guinea pigs were resistant, and like mouse and rabbit histiocytes, this resistance was serum dependent.

TABLE II

Passive Transfer of Cellular Resistance in Mice and Guinea Pigs with Homologous Cells

Type of histiocyte or histiocytic component used for transfer*	No. of cells transferred ($\times 10^6$)‡	Recipient animal§	Infected histiocytes	Serum medium for culturing histiocytes	Average degeneration, 48 hrs. after infection
			per cent		*per cent*
1. Immune M	1.1	Normal M	19	Normal serum	53
2. Immune M	1.1	Normal M	22	Immune serum	0
3. Immune M	118.0	Normal M	22	Normal serum	52
4. Immune M	118.0	Normal M	19	Immune serum	0
5. Normal M	160.0	Normal M	19	Normal serum	47
6. Normal M	160.0	Normal M	20	Immune serum	51
7. Immune M ribosome	210.0	Normal M	20	Normal serum	57
8. Immune M ribosome	210.0	Normal M	21	Immune serum	55
9. Immune M ribosome	1140.0	Normal M	18	Normal serum	56
10. Immune M ribosome	1140.0	Normal M	19	Immune serum	0
11. Immune GP	236.0	Normal GP	21	Normal serum	43
12. Immune GP	236.0	Normal GP	19	Immune serum	47
13. Immune GP	2130.0	Normal GP	14	Normal serum	56
14. Immune GP	2130.0	Normal GP	16	Immune serum	40
15. Normal GP	1000.0	Normal GP	20	Normal serum	46
16. Normal GP	1000.0	Normal GP	18	Immune serum	54
17. Immune GP	0	0	20	Normal serum	58
18. Immune GP	0	0	22	Immune serum	3

* Normal M and normal GP designate normal mouse and guinea pig; immune M and immune GP designate mice and guinea pig immunized with BCG.

‡ Indicates number of histiocytes inoculated intradermally into each recipient animal.

§ Recipient animals were inoculated intradermally with histiocytes or ribosomes; 13 days later the recipient's histiocytes were tested for resistance. For additional explanation of table headings, see footnotes in Table I.

Passive Transfer of Cellular Resistance in Mice and Guinea Pigs with Homologous Cells.—The similarity in behavior of histiocytes from BCG-immunized mice and rabbits is also evidenced by the capacity of immune mouse histiocytes to induce cellular resistance in normal mouse recipients. Table II presents representative data from one of several similar experiments; the upper half of this table clearly shows that 13 days after intradermal injection of normal mice with immune mouse histiocytes, the recipient histiocytes (derived from peritoneal exudates) proved refractory to virulent tubercle bacilli when cultured in immune serum (rows 2 and 4) but not in normal serum (rows 1 and 3). It is

also apparent that while 1.1×10^6 immune histiocytes were adequate for induction of cellular resistance in normal mice (row 2), 160 times as many normal mouse histiocytes failed to effect a similar conversion of recipient histiocytes (row 6).

The data in rows 7 to 10 of Table II present the results of tests with the ribosomes of immune mouse histiocytes. In terms of cell equivalents, it is evident that very large numbers of immune cells are needed to provide enough ribosomes to effect passive transfer of cellular resistance to normal mice; thus, 2.1×10^8 immune histiocytes failed to yield ribosomal transfer of cellular resistance (degeneration of infected recipient histiocytes in rows 7 and 8). When compared with intact mouse histiocytes, the number of cells needed to give ribosomal transfer of resistance was 1000 times greater (compare rows 2 and 10).

In contrast to the positive transfer of cellular resistance to normal mice *via* the agency of homologous immune histiocytes and immune ribosomes, it is evident from the lower half of Table II that the histiocytes of normal guinea pigs could not be made resistant by intradermal injections of immune guinea pig histiocytes (row 18 shows that the histiocytes used for injection of normal guinea pigs were resistant, since cultivation of these cells in immune serum after infection resulted in little destruction of the cells); thus, all of the recipient histiocytes exhibited marked degeneration at 48 hours' postinfection even though immune serum was present (rows 12, 14, 16). This inability to effect passive transfer of cellular resistance in guinea pigs by injections of immune homologous histiocytes is not readily explained in terms of inadequate members of histiocytes transferred since the largest number of cells used in these studies (2.1×10^9 in row 14) was 2000 times that needed for effective homologous transfer in mice, and according to previously reported data, 200 times more than required for successful homologous transfer in rabbits (2). One interpretation of these results would be that despite active induction of cellular resistance in guinea pigs upon immunization with BCG, the cellular modification in guinea pigs is defective in that there is no mechanism for continued perpetuation of the resistant state.

Interspecies Transfer of Cellular Resistance with Rabbit Histiocytes and Histiocytic Ribosomes.—A preceding paper (3) has indicated that the component of immune histiocytes which was active in induction of cellular resistance in recipients was ribosomal ribonucleic acid. In view of the fact that various investigators (6–8) have demonstrated that mammalian cells are capable of taking up homologous as well as heterologous nucleic acids both *in vitro* and *in vivo*, the possibility of interspecies transfer of cellular resistance was suggested. The results of these investigations are presented in this and the two following sections of this report.

Investigations of the ability of immune rabbit histiocytes and histiocytic

TABLE III

Interspecies Transfer of Cellular Resistance with Rabbit Histiocytes and Ribosomes

Type of histiocyte or cellular material used for transfer*	No. cells or cell equiv. transferred ($\times 10^8$), ‡	Recipient animal§	Infected histiocytes‖	Serum for culturing infected histiocytes	Average degeneration	
					24 hrs. after infection	48 hrs. after infection
			per cent		*per cent*	*per cent*
1. Immune R histiocyte	6.7	GP	22	Normal serum	25	59
2. Immune R histiocyte	6.7	GP	21	Immune serum	0	0
3. Immune R histiocyte	64.0	GP	17	Normal serum	31	59
4. Immune R histiocyte	64.0	GP	16	Immune serum	9	9
5. Immune R histiocyte lysate	64.0	GP	20	Normal serum	25	59
6. Immune R histiocyte lysate	64.0	GP	17	Immune serum	15	15
7. Normal R histiocyte	35.0	GP	20	Normal serum	37	59
8. Normal R histiocyte	35.0	GP	18	Immune serum	20	48
9. Immune R histiocyte	2.5	M	21	Normal serum	30	57
10. Immune R histiocyte	2.5	M	19	Immune serum	0	0
11. Immune R ribosomes	3.8	M	20	Normal serum	29	61
12. Immune R ribosomes	3.8	M	19	Immune serum	0	0
13. Immune R ribosomes	23.0	M	14	Normal serum	20	48
14. Immune R ribosomes	23.0	M	12	Immune serum	0	0
15. Normal R histiocyte	4.6	M	19	Normal serum	23	50
16. Normal R histiocyte	4.6	M	16	Immune serum	12	46
17. Normal R ribosomes	4.6	M	17	Normal serum	22	50
18. Normal R ribosomes	4.6	M	19	Immune serum	21	47

For explanation of remaining headings, see Table I.

* Material used for passive transfer consisted of rabbit (R) histiocytes or cellular components of these histiocytes; donors of cells or cellular components were immunized with BCG 30 or more days previously and were tuberculin positive; materials used for transfer experiments were injected intradermally into recipients.

‡ Represents actual numbers of histiocytes or cell equivalents inoculated into recipients (cell equivalents indicate numbers of histiocytes used to prepare the cellular components employed in passive transfer).

§ GP and M designate normal guinea pigs and mice respectively.

‖ Refers to histiocytes or recipient animals which were removed from peritoneal cavity at 13 days post transfer and infected with H37Rv prior to *in vitro* cultivation for testing of cellular resistance.

ribosomes to transfer resistance to normal mice are shown in the lower half of Table III. It is apparent that intradermal inoculation of normal mice with 2.5 $\times$ 10^8 immune rabbit histiocytes resulted in conversion of mouse histiocytes to resistance against the virulent H37Rv strain of tubercle bacillus; as shown in rows 9 and 10, the recipient mouse histiocytes were susceptible to infection in the presence of normal mouse serum and resistant in the presence of homol-

ogous immune serum; whether smaller numbers of immune rabbit histiocytes would induce cellular resistance in normal mice was not tested, since the primary objective was qualitative determination of interspecies transfer of cellular resistance rather than quantitative analysis of the phenomenon. The substitution of immune rabbit histiocytes with immune rabbit histiocytic ribosomes yielded similar results; thus inoculation of normal mice with immune histiocytic ribosomes (equivalent to 3.8 to 23.0 $\times$ 10^8 whole histiocytes) resulted in modification of recipient histiocytes, for no destruction of these cells occurred after their infection and cultivation *in vitro* in the presence of

TABLE IV

Interspecies Transfer of Cellular Resistance with Mouse Histiocytes

Type of histiocyte used for transfer	No. cells transferred ($\times 10^8$)	Recipient animal	Infected histiocytes	Serum for culturing infected histiocytes	Average degeneration, 48 hrs. after infection
			per cent		*per cent*
1. Immune M.....	5.2	R	20	Normal serum	54
2. Immune M.....	5.2	R	22	Immune serum	43
3. Immune M.....	11.8	R	20	Normal serum	51
4. Immune M.....	11.8	R	20	Immune serum	0
5. Normal M.....	25.5	R	19	Normal serum	45
6. Normal M.....	25.5	R	21	Immune serum	53
7. Immune M.....	5.2	GP	18	Normal serum	43
8. Immune M.....	5.2	GP	22	Immune serum	44
9. Normal M.....	25.5	GP	21	Normal serum	52
10. Normal M.....	25.5	GP	20	Immune serum	55

For explanation of table headings, see footnotes of preceding tables.

immune serum (rows 12 and 14). The data of rows 16 and 18 clearly indicate that comparable amounts of normal rabbit histiocytes or histiocytic ribosomes lacked the capacity to induce cellular resistance in mice.

The induction of cellular resistance in normal guinea pigs by intradermal injections of immune rabbit histiocytes and histiocytic lysates is shown in the upper half of Table III. It is evident that the introduction of 6.7 to 64.0 $\times$ 10^8 immune rabbit histiocytes into guinea pigs rendered their histiocytes resistant to virulent tubercle bacilli; this resistance was established either in the entire histiocytic population of the recipient (row 2) or in a predominant portion of the recipient cells (row 4). The injection of immune rabbit histiocytic lysates into normal guinea pigs also led to induction of resistance in the major portion of the recipient's histiocytes. This is evidenced by comparison of the data in rows 5 and 6; whereas there was extensive and progressive destruction of infected recipient histiocytes cultivated in normal homologous serum, there

was only a minor initial and non-progressive loss of infected histiocytes in the presence of immune serum. In contrast, normal rabbit histiocytes failed to induce cellular resistance in guinea pigs (rows 7 and 8).

The induction of cellular resistance in normal guinea pigs by immune rabbit histiocytes (as shown in Table III) but not by immune guinea pig histiocytes (Table II) was an unexpected event. This discrepancy in ability of these two types of histiocytes to induce cellular resistance in normal guinea pigs was not attributable to differences in cell numbers used for transfer. Comparison of the results of Tables II and III reveals that 6.7×10^8 rabbit histiocytes were effective, whereas 2.1×10^9 guinea pig histiocytes proved inadequate; if approximately 10 to 15 per cent of the guinea pig histiocytes used for transfer were ineffective (*i.e.* non-resistant as suggested by the results shown in row 10 of Table I), the number of resistant guinea pig cells introduced into recipients would still exceed that of immune rabbit histiocytes by a factor of 3. It is therefore possible that the different behavior of immune rabbit and immune guinea pig histiocytes is a reflection of fundamental differences in the resistance mechanisms of these cells.

Interspecies Transfer of Cellular Resistance with Mouse Histiocytes.—The results of studies on induction of cellular resistance in normal rabbits and guinea pigs *via* injections of immune mouse histiocytes are shown in Table IV. While histiocytes of normal rabbits can be made resistant by intradermal injections of immune mouse histiocytes, it is apparent that large numbers of immune cells must be used; as shown in rows 2 and 4, 5.2×10^8 immune mouse histiocytes failed to confer cellular resistance upon recipient rabbits, whereas 1.1×10^9 mouse cells were effective. The results of row 6 reveal that normal mouse histiocytes were unable to induce cellular resistance in normal rabbits.

The data in row 8 of Table IV indicate that 5.2×10^8 immune mouse histiocytes were inadequate for induction of cellular resistance in normal guinea pigs. Whether larger numbers of immune mouse histiocytes would prove as effective for guinea pigs as for rabbits was not investigated because of the very large numbers of immunized mice needed to yield the requisite number of cells for additional transfer experiments.

Interspecies Transfer of Cellular Resistance with Guinea Pig Histiocytes.—The results described in the first part of this paper have shown that mouse and guinea pig histiocytes resembled rabbit histiocytes in that the histiocytes of these animal species developed resistance to virulent tubercle bacilli following immunization of animals with BCG; a further point of similarity in the histiocytes of these various animal species was the necessity for immune serum for complete expression of cellular resistance. The results presented in the later sections, however, have indicated a basic difference in the behavior of immune guinea pig histiocytes; thus, unlike either immune rabbit or immune mouse histiocytes which were capable of inducing cellular resistance in either homol-

ogous or heterologous animal species, immune guinea pig histiocytes failed to induce cellular resistance even in the homologous species.

That this defect in homologous transfer (guinea pig to guinea pig) of cellular resistance was attributable to the histiocytes of the donor guinea pig and not to

TABLE V

Interspecies Transfer of Cellular Resistance with Guinea Pig Histiocytes

Type of histiocyte used for transfer*	No. cells transferred ($\times 10^8$)	Recipient animal	Infected histiocytes	Serum for culturing infected cells	Average degeneration	
					24 hrs. after infection	48 hrs. after infection
			per cent		*per cent*	*per cent*
1. Immune GP	20	M	19	Normal M	19	47
2. Immune GP	20	M	21	Immune M	25	48
3. Normal GP	24	M	21	Normal M	17	45
4. Normal GP	24	M	20	Immune M	20	45
5. Immune GP	64	R	18	Normal R	29	61
6. Immune GP	64	R	15	Immune R	10	48
7. Immune GP	16	R (13)‡	15	Normal R	27	56
8. Immune GP	16	R (13)‡	18	Immune R	25	50
9. Immune GP	16	R (28)‡	19	Normal R	34	60
10. Immune GP	16	R (28)‡	20	Immune R	27	56
11. GP (Immune R)§	5.7	GP	20	Normal GP	35	60
12. GP (Immune R)§	5.7	GP	21	Immune GP	10	8
13. GP (Immune R)§	3.2	R	20	Normal R	28	55
14. GP (Immune R)§	3.2	R	21	Immune R	0	0

For additional footnotes, see preceding tables.

* All immune histiocytes used in these transfer experiments were tested and shown to be resistant.

‡ The histiocytes of recipient rabbits were tested for cellular resistance at 13 and 28 days after intradermal injection of immune guinea pig histiocytes.

§ These were peritoneal histiocytes derived from guinea pigs inoculated intradermally 13 days previously with immune rabbit histiocytes.

an inability of recipient guinea pigs to develop cellular resistance after receipt of immune histiocytes was suggested by the development of cellular resistance in guinea pigs following administration of immune rabbit histiocytes (Table III). Further evidence of the basic defectiveness of immune guinea pig histiocytes to effect passive transfer of cellular resistance is shown by the results presented in the upper two-thirds of Table V. These results clearly indicate that the injection of as much as 2.0×10^9 immune guinea pig histiocytes failed to render the histiocytes of normal mice resistant to virulent tubercle

bacilli (rows 1 through 4 show almost identical loss of cells regardless of the history of the recipient histiocytes and the nature of the serum used for cultivation of infected histiocytes). It is equally evident that 1.6 to 6.4 $\times$ 10^9 immune guinea pig histiocytes were unable to induce cellular resistance in normal rabbits (rows 5 through 10); even prolongation of the time between intradermal injection of immune histiocytes and testing of recipient rabbit histiocytes from 13 to 28 days was without effect upon the results (rows 7 through 10). This inability of immune guinea pig histiocytes to induce cellular resistance in these experiments did not result from use of non-resistant cells, for all the cells used in these transfer experiments were tested for resistance and found to be refractory to virulent tubercle bacilli (results of tests for resistance of donor cells not shown in Table V). Since both mice and rabbits are competent recipients (*i.e.* they develop cellular resistance upon receipt of either homologous or heterologous immune histiocytes), the present failure to induce cellular resistance in these two animal species can only be related to an inadequacy of the donor cells.

Inasmuch as it has already been shown that passive transfer of cellular resistance to guinea pigs can be achieved by use of immune rabbit histiocytes, it seemed of interest to determine whether the histiocytes of these recipient guinea pigs were also defective in passive transfer experiments. To test this point, recipient guinea pig histiocytes (from guinea pigs given immune rabbit histiocytes) were used as donor cells and were injected into normal guinea pigs and rabbits; the histiocytes of these two animal species were then tested for cellular resistance at 13 days' posttransfer. As shown in the lower third of Table V, histiocytes of either normal guinea pigs or normal rabbits were rendered resistant to virulent tubercle bacilli by intradermal injection of these animals with recipient guinea pig histiocytes; this is evidenced in the case of the guinea pig by the data of rows 11 and 12 (compare minor and non-progressive degeneration of infected histiocytes in row 12 where immune serum medium was used with marked and progressive destruction of histiocytes in row 11 where normal serum medium was used) and in the case of the rabbit by the data of rows 13 and 14.

Comparison of the results of rows 12 and 14 suggests that the species of animal used as recipient was not completely without effect upon the end-result of cellular transfer of resistance; hence, while the agent of transfer was the same for rabbits and guinea pigs, the conversion of recipient histiocytes was complete in the rabbit but not in the guinea pig (row 12 shows an initial 10 per cent degeneration of cells at 24 hours' postinfection; no degeneration is observed in row 14). The present observation is thus similar to findings recorded in Tables I and III; in all these instances involving induction of cellular resistance in guinea pigs either by immunization or passive transfer of resistant cells, a residuum of unconverted guinea pig histiocytes was occasionally demonstrable.

Cultivation Tests for Viable Bacilli in Donor Histiocytes.—The cumulative evidence for the absence of viable bacilli and bacillary antigens in immune rabbit histiocytes has been presented in a preceding paper (3); on the basis of this body of evidence, the present induction of cellular resistance in normal mice and guinea pigs *via* intradermal injection of these animals with immune rabbit histiocytes or histiocytic ribosomes is attributable to an active subcellular component of the immune cell and not to viable bacilli or bacillary antigens. Transfer of cellular resistance to normal rabbits and guinea pigs by use of recipient guinea pig histiocytes (from guinea pigs given immune rabbit histiocytes) is similarly interpretable in terms of induction of resistance by a material of host cell origin. In fact, these experiments, in which guinea pig donor cells were used, constitute additional and cogent evidence against the contention that viable bacilli are required for induction of resistance in recipient animals; thus, if viable bacilli were involved, it becomes most difficult to explain the positive transfers with recipient guinea pig histiocytes and the negative transfers with immune guinea pig histiocytes where carry-over of bacilli by histiocytes is more likely because of the large numbers of living bacilli used for immunization.

Despite the various earlier negative tests for presence of living bacilli in donor histiocytes, it was deemed desirable to test for viable bacilli in still another way. For this purpose, immune donor histiocytes (from immunized rabbits, mice, and guinea pigs) were lysed in distilled water and the entire lysate cultivated in plates of glycerol-blood agar medium (9). The results (not presented in tabular form) indicated that *in vitro* cultivation of lysed immune histiocytes (9.7×10^9 rabbit histiocytes; 5.5×10^{10} guinea pig histiocytes; 1.1×10^8 mouse histiocytes) failed to yield any colonies of BCG. As shown by the data presented in this and an earlier paper (2), these amounts of histiocytes which were subjected to cultivation tests were greatly in excess of the amounts needed to effect transfer of cellular resistance in homologous animal species (1.0×10^6 for mouse to mouse transfer and 1.0×10^7 for rabbit to rabbit transfer); in most instances where successful transfer occurred in heterologous species, the number of histiocytes needed for induction of resistance was also 10 to 100 times less than the number used in tests for viable bacilli (2.5 and 6.7×10^8 cells for transfer from rabbits to guinea pigs and mice respectively; 3.2 and 5.7×10^8 for transfer from recipient guinea pigs to normal rabbits and guinea pigs respectively); the single exception was the transfer of resistance from mouse to rabbit where approximately 1.0×10^9 cells were needed (whether this amount of mouse cells would have yielded colonies of BCG is not known since only 1.0×10^8 mouse cells were cultivated).

DISCUSSION

These studies have investigated the effects of immunization upon the histiocytes of the mouse and the guinea pig and have shown that induction of cellular

resistance is an event common to both animal species. Since earlier studies have demonstrated that immunization of rabbits with BCG also resulted in development of histiocytic resistance against virulent tubercle bacilli (1), a certain similarity in the cellular response of all three animal species was indicated. Further examination of this problem, however, revealed the fact that the immune mouse histiocyte (from BCG-immunized mouse) was functionally more closely related to the immune rabbit histiocyte than to the immune guinea pig histiocyte. Functional similarity of immune mouse and immune rabbit histiocytes was evidenced by the fact that both types of histiocytes were capable of passively transferring resistance to either homologous or heterologous animal species. In contrast, immune guinea pig histiocytes failed to induce cellular resistance in either category of animals.

Although immune guinea pig histiocytes proved totally ineffective in transfer experiments, this was not the case with recipient guinea pig histiocytes (from guinea pigs inoculated 13 days previously with immune rabbit histiocytes). These latter cells resembled immune mouse and rabbit histiocytes in their ability to effect both homologous and heterologous (recipient guinea pig cells inoculated into normal guinea pigs or rabbits) transfers of cellular resistance.

The above observations thus allow for functional separation of resistant histiocytes into two categories: (*a*) resistant histiocytes such as immune guinea pig histiocytes which lack the capacity for inducing cellular resistance in normal animals, and (*b*) resistant histiocytes such as immune mouse, immune rabbit, and recipient guinea pig histiocytes, which are capable of transferring resistance to normal animals.

The present investigations have not attempted to analyze the mechanisms involved in induction of cellular resistance by the second category of cells listed above. Earlier studies (3) have shown, however, that homologous induction of cellular resistance in rabbits is related to ribosomal ribonucleic acid, and it seems reasonable to postulate a similar mechanism for the various types of homologous and heterologous transfers which were reported herein. This assumption of identity of mechanism for induction of cellular resistance is based in part on the functional similarity of these cells and in part on the fact that in the two instances where histiocytic ribosomes were tested (in homologous mouse to mouse and in heterologous rabbit to mouse transfers), they were found to be active. More definitive proof requires studies along lines similar to those previously described for rabbit ribosomes (3).

The basic defect of immune guinea pig histiocytes which renders them incapable of effecting transfer of cellular resistance is most likely due to the absence of a mechanism for perpetuation of the resistance factor; the earlier demonstration of the importance of ribosomal ribonucleic acid in induction of cellular resistance (3) and the observation that serial transfer of cellular resistance is possible with histiocytic ribosomes (results to be reported) would

suggest a deficiency in production of the appropriate ribonucleic acid by immune guinea pig histiocytes.

An important question which has been posed as a result of these studies is one which is concerned with the reasons for the basic difference in the histiocytes of immunized rabbits and guinea pigs. Inasmuch as *in vivo* differences between rabbits and guinea pigs to infection with human tubercle bacilli do exist, it is tempting to speculate on the possible correlation of the present *in vitro* differences with the known *in vivo* responses of these two animals. This whole question of the significance of these *in vitro* findings for the intact animal is presently under intensive investigation.

The contention that induction of cellular resistance in these experiments is due to viable bacilli or bacillary antigens can in a large measure be nullified by accumulation of a large body of contrary evidence. The nature of this evidence has been summarized in a recent publication (3); the present negative results of attempts to demonstrate viable BCG by cultivation of disrupted histiocytes can now be added to this growing body of evidence against the concept of induction of cellular resistance by bacilli.

SUMMARY

Immunization of mice or guinea pigs with BCG rendered all or most of the histiocytes of these animals resistant to necrotization by virulent H37Rv; this cellular resistance was mediated by immune serum.

Immune mouse histiocytes (from BCG-immunized animals) were able to induce cellular resistance in normal homologous and heterologous (rabbit) animal species; mouse histiocytic ribosomes were also tested in the homologous species and found to be active.

Immune guinea pig histiocytes (from BCG-immunized guinea pigs) were ineffective in transferring cellular resistance to either homologous or heterologous (mouse and rabbit) animal species.

Immune rabbit histiocytes were capable of inducing cellular resistance in mice and guinea pigs; rabbit histiocytic ribosomes were also tested in normal mice and found to be active in induction of cellular resistance.

Recipient guinea pig histiocytes (from guinea pigs inoculated with immune rabbit histiocytes) were capable of inducing cellular resistance in normal guinea pigs and rabbits.

Cultivation of lysed immune histiocytes of all three animal species on glycerol-blood agar medium failed to reveal any viable BCG; this provided one additional bit of evidence against the idea that induction of cellular resistance is due to viable bacilli.

BIBLIOGRAPHY

1. Fong, J., Schneider, P., and Elberg, S. S., Studies on tubercle bacillus–histiocyte relationship. I. Quantitative analysis of effect of serum of animals vaccinated with BCG upon bacterium-monocyte system, *J. Exp. Med.*, 1956, **104**, 455.

2. Fong, J., Chin, D., and Elberg, S. S., Studies of tubercle bacillus–histiocyte relationship. V. Passive transfer of cellular resistance, *J. Exp. Med.*, 1962, **115,** 475.
3. Fong, J., Chin, D., and Elberg, S. S., Studies of tubercle bacillus–histiocyte relationship. VI. Induction of cellular resistance by ribosomes and ribosomal RNA, *J. Exp. Med.*, 1963, **118,** 371.
4. Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C., Studies on cytoplasmic ribonucleoprotein particles from the liver of the rat, *J. Biol. Chem.*, 1955, **217,** 111.
5. Mackaness, G. B., The action of drugs on intracellular tubercle bacilli, *J. Path. and Bact.*, 1952, **64,** 429.
6. Niu, M. C., Cordova, C. C., and Niu, L. C., Ribonucleic acid–induced changes in mammalian cells, *Proc. Nat. Acad. Sc.*, 1961, **47,** 1689.
7. Amos, H., Protamine enhancement of RNA uptake by culture chick cells, *Biochem. and Biophysic. Research Commun.*, 1961, **5,** 1.
8. Schwarz, M. R., and Rieke, W. O., Appearance of radioactivity in mouse cells after administration of labeled macromolecular RNA, *Science*, 162, **136,** 152.
9. Tarshis, M. S., and Elberg, S. S., Enumeration of viable tubercle bacilli, *Am. Rev. Tuberc.*, 1956, **74,** 84.