STUDIES ON TUBERCLE BACILLUS-MONOCYTE RELATIONSHIP*

III. CONDITIONS AFFECTING THE ACTION OF SERUM AND CELLS. MODIFICATION OF BACILLI IN AN IMMUNE SYSTEM

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Although various observations (1–5) have failed to relate serum antibody to resistance against tuberculosis, these findings need not preclude a role for humoral factors in an as yet indeterminate and possibly rather complex pattern of resistance. The participation of humoral factors in resistance to tubercle bacilli was indicated by the observation that the sera of animals immunized with the BCG strain of tubercle bacillus contained a substance which protected the monocytes of these immunized animals against the necrotizing action of virulent tubercle bacilli (6). While the protective substance was found in immune and not in normal sera, its activity was seemingly non-specific in nature, for the immune sera of animals immunized with antigens totally unrelated to the tubercle bacillus (*Salmonella*, *Brucella*, ovalbumin) proved equally effective in protecting the monocytes of BCG-immunized animals against virulent tubercle bacilli (7, 8). It was also found that these immune sera protected the monocytes of the BCG-immunized animal but failed to afford similar protection to normal monocytes (6).

Studies of cellular resistance showed that it was also not entirely specific, since, in the presence of immune sera, the monocytes of animals immunized with either *Mycobacterium* or *Brucella* proved partially or completely resistant to heterologous as well as homologous infection (8). Despite the seeming lack of specificity suggested by the cross-immunity between the cells of animals immunized with *Brucella* and *Mycobacterium* some selectivity of action was demonstrable in cells; thus, while both anti-*Brucella* and anti-*Salmonella* sera contained a substance which protected the cells of BCG-immunized animals against virulent tubercle bacilli, the monocytes of the *Brucella*- and *Salmonella*-immunized animals behaved quite differently in that the cells of the *Salmonella*-immunized animal proved completely susceptible to virulent tubercle bacilli (7).

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It is therefore evident that effective resistance against tubercle bacilli involved an interacting system of immune serum and immune cells and that this system is subject to a number of permutations. Accordingly, any study of the mechanism of resistance to tubercle bacilli should include not only analyses of the individual role of cells and serum, but also investigations of the combined effects of both upon virulent tubercle bacilli.

The present report deals with additional studies of the specificity of serum action, conditions affecting the activities of serum and cells, and the synergistic effects of serum and cells upon virulent tubercle bacilli.

Materials and Methods

Complete details are given for those materials and methods not heretofore utilized; those which were previously used (6, 7) are briefly described in this report.

Monocytes.—Normal adult rabbits and rabbits injected intradermally with viable BCG or heat-killed BCG were used as monocyte donors. The time of collection of monocytes was either 5 days or 30 or more days after injection of the test substance.

The monocytes were obtained by washing the peritoneal cavity of rabbits with 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 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 freshly collected normal or immune (BCG-immunized) rabbit serum, and the number of cells present was determined by counting in a hemocytometer. These cells were then parasitized with virulent tubercle bacilli in the manner described below.

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

For use in parasitization of monocytes, 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 dark-ground illumination, the supernatant fluid was used as a source of bacteria in parasitization of monocytes.

In the absorption of anti-BCG serum, a strain of H37Rv which had been grown on glycerol-blood agar medium (9) and collected over a period of several months was used. The bacteria were killed by heating at 60°C. for 30 minutes. The heated bacterial suspension was washed several times in physiological saline and the washed bacterial cells were resuspended in a small volume of diluent to yield a thick suspension containing 1×10^{10} or more bacteria per ml.

The BCG strain of tubercle bacillus was cultivated on Calmette's potato medium. For use in injection of animals which were employed as monocyte and serum donors, 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. The amount of viable bacteria injected was 0.1 to 0.2 ml.; the amount of heat-killed (60°C. for 30 minutes) bacteria injected was 1.0 to 2.0 ml. distributed over several sites in the skin.

Salmonella rutgers was cultivated for 18 to 24 hours at 37°C. in a nutrient agar medium contained in large flat bottles. The bacterial growth was washed several times in physiological

saline and resuspended in a small volume of diluent; an equal volume of 0.6 per cent formalin was added. Inactivation of the bacteria was carried out at 37°C. The inactivated bacterial suspension was adjusted, by addition of formalinized saline, to yield a suspension containing 9.0×10^{10} bacteria per ml. For absorption of anti-*Salmonella* serum, the formalinized suspension was used undiluted; for immunization of rabbits the inactivated bacterial suspension was diluted 1:10 in physiological saline; for agglutination tests the bacterial suspension was diluted 1:100.

Old Tuberculin.—The usual commercially available O.T. was used in skin sensitivity tests.

Normal, Immune, and Miscellaneous Sera.—Normal serum consisted of the pooled sera of adult, tuberculin-negative rabbits.

Anti-BCG serum consisted of aged, pooled sera from tuberculin-positive rabbits injected intradermally with 0.1 to 0.2 ml. of viable BCG cells 30 or more days previously.

The 5-day sera were obtained from animals 5 days after injection of either 0.1 to 0.2 ml. of viable BCG cells or 1.0 ml. of heat-killed BCG cells. The use of these sera is indicated in Table IV of this report.

Heated serum consisted of anti-BCG serum exposed to 60° or 70°C. for 30 minutes in a water bath.

The dialysis of anti-BCG serum was carried out in cellophane bags; 30 ml. of immune serum was dialyzed against normal serum medium for 7 days with daily changes of serum medium (120 ml. per day). Normal serum medium was used rather than Tyrode's solution alone since dialysis of serum against the balanced salt solution yielded sera which failed to maintain *in vitro* cultures of monocytes.

Anti-*Salmonella* serum represented the aged, pooled sera of rabbits immunized with *Salmonella rutgers*. Immunization consisted of 6 injections of *Salmonella* antigen administered on alternate days in the manner previously described (7).

Absorption of Sera.—Anti-*Salmonella* serum was absorbed with 3 successive portions of the absorbing antigen prepared as described above. Each absorption was carried out for 1 hour at 37°C. After the last absorption, no agglutination was observed when dilutions of the absorbed serum were tested with the agglutinating suspension of *Salmonella rutgers*. Tests for presence of non-agglutinating antibody with 20 per cent solution of plasma albumin in saline as diluent also proved negative.

Anti-BCG serum was absorbed with 3 successive portions of the heat-killed H37Rv strain prepared as described above. The absorbed serum was not tested for residual agglutinins before use.

Serum Globulin.—Immune serum globulin was prepared from pooled anti-BCG serum. Globulin was precipitated by mixing equal volumes of serum and saturated ammonium sulfate solution. The precipitate was removed by centrifugation, redissolved in water, and reprecipitated with ammonium sulfate. The second precipitate of globulin was dissolved in Tyrode's solution, placed into a collodion sac and dialyzed in the cold against Tyrode's solution for 4 to 7 days. The globulin preparation was not utilized until preliminary tests had shown that the preparation was no longer toxic for *in vitro* cultures of rabbit monocytes. Before use, enough Tyrode's solution was added to the dialyzed globulin preparation to restore its volume to that of the original serum used in its preparation.

Normal globulin was prepared in the same manner from normal rabbit serum except that after dialysis the globulin solution was restored to ¾ of the original volume of serum used. This was to compensate in part for the smaller amounts of globulin in normal as opposed to immune serum.

Nutrient Media.—The medium used for cultivation of monocytes consisted of 40 per cent rabbit serum (normal, anti-BCG, or anti-*Salmonella*) in Tyrode's solution. The particular type of serum used in an experiment is generally indicated in the table which summarizes the results of the experiment.

In experiments designed to test the ability of globulin to protect immune monocytes against virulent tubercle bacilli, the solution of normal or immune globulin, prepared as described above, was used in place of Tyrode's solution. Enough normal serum was added to yield a medium consisting of 40 per cent serum in globulin solution.

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

Parasitization of Monocytes.—The H37Rv strain of tubercle bacillus was mixed with the monocytes (obtained from animals injected with the various test substances described under Monocytes) in a ratio of approximately 10 bacteria per monocyte; 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 the desired nutrient medium. The number of monocytes was determined in a hemocytometer, and the infected monocyte suspension was diluted with additional nutrient medium to yield approximately 15 monocytes per c.mm.; the diluted suspension of infected cells was used for *in vitro* cultures as described below.

Cultivation of Monocytes.—This was carried out in the culture chambers described by Mackaness (10). Approximately 0.05 ml. of diluted infected monocyte suspension was introduced into the space delineated by a plastic ring affixed to the bottom coverslip of the culture chamber. This yielded about 500 to 1000 cells in the culture chamber. After adherence of monocytes to the bottom coverslip, the culture chamber was closed by insertion of the top coverslip. Sufficient nutrient medium of the desired type 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 monocytes 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 (6).

Staining of Preparation on Coverslips.—When it was desired to establish the proportion of infected monocytes or the number of ingested bacteria, a coverslip with a centrally affixed plastic ring was prepared, and a small amount of the parasitized cell suspension was introduced into the central area. 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 method.

Percentage of Infected Monocytes.—This was determined by counting a total of 200 stained cells.

Average Number of Bacteria per Infected Monocyte.—This was obtained by examining 200 stained cells, counting the total number of intracellular bacteria, and dividing this total by the number of infected monocytes.

Determination of Viable Bacteria in Infected Monocyte Cultures.—In experiments involving determination of viable bacteria in monocyte cultures, the infected monocytes were cultivated in Carrel flasks. The procedure for parasitization of monocytes was as described previously except that after parasitization the infected monocytes were washed once with 10 ml. of Tyrode's solution to remove the bulk of any extracellular bacteria not taken up by phagocytes. The infected monocytes were resuspended in the desired nutrient medium and 20,000 to 40,000 infected cells were introduced into Carrel flasks. The number of bacteria present in the Carrel flasks was determined at the start of the experiment and after varying periods of incubation at 37°C. For each interval of time, the contents of three Carrel flasks were pooled and used. Lysis of the monocytes was achieved by adding enough saponin to the contents of the Carrel flasks to yield a final concentration of 2 per cent saponin. This concentration was adequate to lyse monocytes and effect release of intracellular bacteria without causing inactivation of the bacteria. The entire content of the Carrel flask was subjected to repeated pipetting to effect disaggregation of bacterial clumps as well as to facilitate lysis of cells. The

sample was then examined microscopically for evidence of aggregation (samples treated in this manner mostly consisted of single bacterial cells). Dilutions of the sample were made in tween-albumin liquid medium and each dilution was added in triplicate to the surface of each of 4 glycerol-blood agar plates by the drop method (9). The plates were incubated for 12 to 14 days at 37°C., and those plates which contained suitable numbers of colonies were counted and the average of all 4 plates calculated.

Parallel cultures were generally set up in the Mackaness-type culture chambers to allow correlation of cellular degeneration with bacterial multiplication.

EXPERIMENTAL

Survival of Infected Immune Monocytes in Presence of Absorbed Sera.—It has been reported (6) that under appropriate conditions of *in vitro* cultivation, the

TABLE I

Survival of Infected Immune Monocytes in the Presence of Absorbed Immune Sera

Type of monocyte	Serum used in cultivation of monocytes	Per cent infected monocytes	Per cent degeneration* (hrs. after incubation)
			48
Immune‡	Normal rabbit serum	14	23
"	Anti-BCG serum	"	1
"	Absorbed anti-BCG serum§	"	3
"	Anti-*Salmonella* serum	"	2
"	Absorbed anti-*Salmonella* serum‖	"	0

* Represents average per cent degeneration (from initial count) in 2 replicate cultures.

‡ Monocytes obtained from tuberculin-positive rabbits immunized with the BCG strain 30 or more days previously. The immune monocytes were parasitized in the presence of the same serum as that subsequently used for their cultivation.

§ Absorbed with washed, whole, heat-killed H37Rv strain.

‖ Absorbed with washed, whole, formalin-killed *Salmonella rutgers*.

monocytes of rabbits immunized with the BCG strain of tubercle bacillus were resistant to the necrotizing action of virulent tubercle bacilli. One of the determinants in protection of these monocytes was immune serum; since it was subsequently observed (7, 8) that either homologous (serum of animals immunized with the BCG strain of tubercle bacillus) or heterologous (sera of animals immunized with *Salmonella*, *Brucella*, egg albumin) immune sera could be used for this purpose, it was suggested that the action of serum factor was entirely non-specific. This problem has been pursued further and the results obtained with absorbed sera and the globulin fraction of immune serum are described in this and the next section of the present report.

The data shown in Table I confirm the earlier observations concerning the protective effects of homologous and heterologous immune sera; whereas the number of immune cells which had been infected with the H37Rv strain of

tubercle bacillus and cultivated in the presence of normal serum had decreased 23 per cent in a period of 48 hours, similarly infected cells maintained in an environment containing unabsorbed anti-BCG or anti-*Salmonella* serum showed less than a 5 per cent drop in cell population.

The absorption of an anti-*Salmonella* serum with homologous antigens and an anti-BCG serum with the antigens of a washed, heat-killed strain of H37Rv failed to alter the protective capacity of these sera, as evidenced by the absence of significant monocytic degeneration in the test systems 48 hours after parasitization.

The absence of residual antibody either of the agglutinating or nonagglutinating variety in absorbed anti-*Salmonella* serum was established in preliminary tests (results not shown). In view of its protective activity in these experiments,

TABLE II

Survival of Infected Immune Monocytes in Immune Globulin Medium

Type of monocyte	Medium used in cultivation of monocytes	Per cent infected monocytes	Per cent degeneration* (hrs. after incubation)	
			24	48
Immune‡	Normal serum	35	32	35
"	Immune "	35	2	9
"	Normal globulin§	35	32	37
"	Immune "§	35	2	30

* Represents average per cent degeneration in 2 replicate cultures.

‡ Immune monocytes were parasitized in the presence of the same medium as that subsequently used for their cultivation.

§ These media contain 40 per cent normal rabbit serum in globulin solution.

it would appear that there was no close association of protective serum factor and antibody globulin. Although tests for residual antibody were not made with the absorbed anti-BCG serum, it seems rather unlikely that much antibody would be left after successive cycles of absorption with the antigens of a closely related strain of tubercle bacillus.

Survival of Infected Immune Monocytes in the Presence of Immune Globulin.—The apparent lack of specific association of protective serum factor with antibody globulin is further substantiated by the data of Table II which presents one of a number of similar experiments depicting the survival of infected immune monocytes cultivated *in vitro* in the presence of immune globulin.

The results indicate that there was some delay in the onset of degeneration in infected monocyte cultures in the presence of immune globulin; the less than 5 per cent degeneration of infected immune monocytes in immune globulin during the first 24 hours of monocyte-bacterium interaction compared favorably

with control cultures of infected immune monocytes cultivated in immune serum. In contrast, infected immune monocytes which were cultivated in normal serum medium showed a 32 per cent decrease in cell numbers. The action of immune globulin did not, however, extend over a 48 period, since the infected immune monocytes exhibited at this time an average degeneration of 30 per cent. Although infected cells cultivated in immune serum showed a slight loss of cells after 48 hours (9 per cent), there can be little doubt that whole immune serum proved much more effective in protecting immune cells against virulent tubercle bacilli than immune globulin. It may be noted that the percentage of infected monocytes was fairly high (35 per cent) and might account for the

TABLE III

Effect of Dialysis and Heat on Protective Factor in Immune Serum

Type of monocyte*	Serum used in cultivation	Per cent infected monocytes	Per cent degeneration of monocytes (hrs. after incubation)‡	
			24	48
Immune	Heated 60°C.§	22	2	5
"	" 70°C.§	22	0	1
"	Dialyzed‖	21	0	2
"	Normal	22	30	47
"	Immune	23	3	4

* Monocyte donors were tuberculin-positive rabbits immunized with BCG 30 or more days previously.

‡ Average per cent degeneration (in two or more culture chambers) from original monocyte count.

§ Heating of immune serum was at the desired temperature for 30 minutes.

‖ Dialysis of immune serum was against normal serum medium for 7 days with daily changes of normal serum medium.

Uninfected monocyte cultures showed less than 5 per cent degeneration in all cases after 48 hours of incubation.

slight degeneration of immune monocytes cultivated in immune serum; in other experiments (results not shown) in which the percentage of infected monocytes was lower (15 to 20 per cent), degeneration occurred in cells which were cultivated in immune globulin but not in whole immune serum.

The delayed degeneration of infected immune monocytes in immune globulin was not apparent when the infected immune cells were cultivated in a normal globulin medium as evidenced by the 32 per cent degeneration of these cells after 24 hours of monocyte-bacterium interaction.

Effect of Dialysis and Heat on Protective Serum Factor.—The effect of heat and dialysis upon the protective factor in immune serum is shown in Table III. It is evident from the per cent degeneration of infected monocytes (5 per cent

or less at 24 and 48 hours after infection) that heating of immune serum at 60–70°C. failed to destroy the protective serum factor.

It may also be observed in Table III that dialysis of immune serum did not remove the protective factor as infected immune monocytes maintained in the presence of a nutrient medium consisting of dialyzed immune serum and Tyrode's solution likewise showed less than 5 per cent loss of monocytes.

The control samples in Table III yielded the usual results, namely, degeneration of infected monocytes in the presence of normal serum but not in immune serum.

Relationship of Protective Serum Factor to Cellular Resistance and Tuberculin Skin Sensitivity.—The earlier investigations (6, 7) have shown that 30 or more days after immunization of rabbits with the BCG strain of tubercle bacillus, their monocytes became immune in the sense that they proved highly resistant to the necrotizing action of virulent tubercle bacilli; however, complete expression of this resistance at the cellular level required mediation by a protective factor present in the sera of the immunized animals. Since these earlier studies utilized only those cells and sera which had been obtained from animals exhibiting a concurrent tuberculin skin sensitivity, it has not been possible to establish the relationship of each of these three factors (protective serum factor, cellular resistance and tuberculin hypersensitivity) to one another. The investigations which are described in this section were, therefore, designed to elucidate the nature of this relationship.

In these experiments rabbits were injected intradermally with a viable BCG strain or a heat-killed BCG strain of tubercle bacillus. The sera and monocytes of these animals were collected 5 days after injection of the test substance, and the tuberculin skin sensitivity of the animals was recorded at the same time. The 5-day sera were tested for presence of protective factor by infecting the monocytes of an immunized animal (monocytes of tuberculin-positive rabbits immunized with the BCG strain 30 or more days earlier) with the virulent H37Rv strain and cultivating these infected immune monocytes in a nutrient medium containing the test serum. The presence or absence of protective factor in these 5-day sera will then be reflected in the per cent degeneration of the infected immune monocytes. The 5-day monocytes were tested for development of cellular resistance by infecting them with the virulent H37Rv strain and cultivating them in a nutrient medium prepared from a pool of anti-BCG serum known to contain a protective factor.

It may be seen in Table IV that intradermal injection of rabbits with a viable BCG strain or a heat-killed BCG strain of tubercle bacillus stimulated the appearance of a protective factor in their sera within 5 days after administration of the test substance. This was evidenced in the average per cent degeneration of monocytes; infected immune cells cultivated in each of the 5-day sera exhibited less than 5 per cent degeneration at both 24 and 48 hours after infection.

Since a value of 5 per cent is within the limits of error of the method of monocyte enumeration, it may be concluded that the concentration of protective factor in each of the sera was sufficient to protect immune monocytes against virulent tubercle bacilli.

Studies of the development of cellular resistance in the same series of animals revealed that the 5-day monocytes of animals injected with a viable strain of BCG possessed a level of cellular resistance which protected them against the virulent H37Rv strain. In contrast to the less than 5 per cent degeneration of

TABLE IV

Relationship of Protective Serum Factor to Cellular Resistance and Tuberculin Sensitivity

Immunizing agent	Amount injected	Tuberculin reaction*	Test system‡	Per cent infected monocytes	Per cent degeneration§ (hrs. after incubation)	
					24	48
Live BCG	2.0×10^7	Negative	5-day serum + immune cells + H37Rv	19	0	3
Killed BCG‖	40.0×10^7	"	" "	19	2	3
Live BCG	2.0×10^7	"	5-day cells + immune serum + H37Rv	18	0	4
Killed BCG‖	40.0×10^7	"	" "	15	24	31
Live BCG	2.0×10^7	Positive	Immune cells + normal serum + H37Rv	17	22	27
" "	"	"	Immune cells + immune serum + H37Rv	19	0	0

* Tuberculin reaction at time of removal of serum and monocytes.

‡ The 5-day serum was incorporated into the nutrient medium used for cultivation of monocytes; the immune cells and immune sera were obtained from tuberculin-positive rabbits injected with live BCG 30 days earlier.

§ Represents average per cent degeneration (from initial count of approximately 500 to 1000 monocytes per culture chamber) in 2 to 3 replicate cultures.

‖ Killed by heating at 60°C. for 30 minutes.

these infected monocytes, it may be observed that 31 per cent of the monocytes derived from animals injected with heat-killed BCG had been destroyed 48 hours following infection.

Control samples consisting of infected immune monocytes suspended in normal serum or in immune serum are included in Table IV. These exhibited the expected results (27 and 0 per cent average degeneration respectively at 48 hours).

It is also apparent in Table IV that the tuberculin response was negative in all animals at the time of withdrawal of the 5-day sera and monocytes.

Other similar experiments have exhibited the same general trend as that

shown in Table IV. It is evident from these results that evocation of the protective serum factor can be achieved with relative facility and that its appearance may not necessarily be correlated with the existence of a high level of cellular resistance. It would seem, moreover, that evolution of tuberculin hypersensitivity, as manifested by a positive skin reaction, was not an essential condition for either development of cellular resistance or production of protective serum factor.

Effect of Serum Treatment upon Capacity of H37Rv to Induce Monocytic Degeneration and to Multiply Intracellularly.—The resistance exhibited against virulent tubercle bacilli by immune cells in the presence of immune serum may reside in one or several possible mechanisms. Some possible modes of action are (*a*) a direct action of immune serum upon virulent tubercle bacilli with consequent modification of its behavior towards the cell, (*b*) an indirect effect of immune serum upon cells leading to cellular alterations that permit continued survival of bacteria in cells without destruction of the cells, and (*c*) modification of the bacterial cell resulting from the combined action of serum and cells. These various alternatives have been studied and the results are presented in the sections which follow.

An obvious mode of action of immune serum would be a direct effect of serum upon the bacteria. Two consequences of such direct action of serum upon bacteria may be modification of the bacterial potential for induction of monocytic degeneration and for proliferation within monocytes. These possibilities were investigated by exposing the virulent H37Rv strain of tubercle bacillus to normal or immune (anti-BCG) serum and by testing the ability of the serum-treated bacteria to destroy immune monocytes and to proliferate in monocytes.

The bacteria were prepared according to the procedure described under Materials and Methods; equal volumes of bacteria and undiluted normal or immune serum were mixed and kept at 37°C. for 15 hours. The serum-treated bacteria were used to parasitize immune monocytes. After parasitization the infected monocytes were washed twice in Tyrode's solution. The washed cells were resuspended in normal serum medium and counted in a hemocytometer. The cells were diluted to contain 15 cells per c.mm. and cultured in a Mackaness-type culture chamber. For determination of viable bacteria, parallel cultures were prepared in Carrel flasks and used as described under Materials and Methods. Since it was desired to establish whether immune serum exerted a direct action upon virulent tubercle bacilli *in vitro*, it was necessary to avoid introduction of immune serum into the tissue culture system after infection of the monocytes; accordingly, monocytes infected with serum-treated bacteria were cultivated in normal serum medium. The results of these experiments are presented in Table V.

As might be expected, *in vitro* exposure of virulent tubercle bacilli to normal serum resulted in little change in the ability of the bacteria to cause monocytic degeneration, for approximately 35 per cent of the immune monocytes infected with normal serum-treated bacteria and cultivated in normal serum medium

were destroyed during a 48 hour period. *In vitro* treatment of virulent tubercle bacilli with immune serum likewise failed to prevent induction of monocytic degeneration; the reduction in cell population for immune monocytes infected with immune serum-treated bacteria equalled that of the monocytes infected with normal serum-treated bacteria. By way of contrast, there was effective protection of monocytes when the infected immune monocytes were cultivated in the presence of immune serum.

The results of investigations of the effect of serum treatment upon the capacity of virulent tubercle bacilli to multiply in immune monocytes are also shown in Table V. It may be seen that when immune monocytes were infected and

TABLE V

Effect of Serum Treatment upon Capacity of H37Rv to Induce Monocytic Degeneration

Treatment of H37Rv*	Type of monocytes infected‡	Medium used in cultivation of infected monocytes	Per cent degeneration§ (hrs. after incubation)	Bacterial fold-increase (hrs. after incubation)
			48	48
Exposed to normal serum	Immune	Normal serum medium	35	6.0
" " " "	"	Immune " "	5	5.1
Exposed to immune serum	"	Normal " "	37	5.7

* The H37Rv strain was exposed to serum for 15 hours at 37°C. prior to use for infection of monocytes.

‡ Monocytes from tuberculin-positive rabbits immunized with BCG 30 or more days previously.

§ Control (uninfected) monocytes in normal or immune serum medium showed less than 5 per cent degeneration in 48 hours (results not shown in table). The values shown in the table represent average (of two or more culture chambers) per cent degeneration in monocyte cultures.

maintained in normal serum medium, the increase in bacterial concentration 48 hours later was 6-fold for bacteria previously exposed to normal serum and 5.7-fold for bacteria treated with immune serum.

As a further point of interest, it may be seen that the increase in bacterial concentration in immune monocytes cultivated in immune serum medium, in which there was no degeneration of cells, was 5.1-fold. An obvious inference of this observation would be that an increase in bacterial cell numbers within infected monocytes needs not necessarily result in destruction of the monocytes.

It was not established in these experiments whether the increases in bacterial concentrations resulted from intracellular, extracellular, or a combination of both types of bacterial proliferation. It should be noted, however, that one consequence of degeneration of infected immune monocytes in normal serum medium is accumulation of extracellular bacteria; the fact that the bacteria

increase under these circumstances was nearly identical with that of monocyte cultures in immune serum (in which bacterial multiplication would primarily be intracellular since there was no degeneration of cells) would seem to suggest that the bacterial increase in these immune monocyte cultures was mainly intracellular. On this basis, it does not seem reasonable to explain the bacterial reproduction in monocyte cultures in terms of superimposition of extracellular upon intracellular multiplication; moreover, inoculation of either normal serum medium or immune serum medium (without monocytes) with 1×10^5 bacteria per ml. failed to exhibit any increase in bacterial concentration after 48 hours of incubation (results not shown in table).

TABLE VI

Resistance of Normal and Immune Monocytes after in Vitro Cultivation in Normal and Immune Sera

Type of monocyte*	Serum used for prior cultivation of monocytes‡	Cultivation serum after infection of monocytes	Per cent of infected cells	Per cent degeneration§ (hrs. after incubation)	
				24	48
Normal	Normal	Immune	21	19	28
"	Immune	"	17	29	33
Immune	Normal	"	15	3	3
"	Immune	"	19	4	5

* Monocytes were derived from normal rabbits (tuberculin-negative) or rabbits immunized with BCG 30 or more days previously (tuberculin-positive); the monocytes were trypsinized and washed before cultivation.

‡ Serum was derived from tuberculin-negative or BCG-immunized, tuberculin-positive rabbits; aged serum was used to make up 40 per cent serum-Tyrode medium for *in vitro* cultivation of the monocytes.

§ Represents average per cent degeneration of monocytes in two or more culture chambers.

These results indicate, therefore, an inability of immune serum to affect directly either the capacity of virulent tubercle bacilli to induce degeneration of monocytes or their ability to multiply intracellularly.

Effect of Prior Cultivation of Monocytes in Normal and Immune Sera upon Their Capacity to Resist Virulent Tubercle Bacilli.—The possibility that immune serum may indirectly influence the ability of virulent tubercle bacilli to cause degeneration of monocytes by modification of the monocyte in the direction of an increased resistance to necrotization by virulent bacilli may be tested rather simply.

One of several experiments designed to determine such indirect effect of immune serum on cells is shown in Table VI; monocytes (normal or immune) were cultivated *in vitro* in the presence of serum (normal or immune) for a period of 24 hours; these monocytes were then treated in the same manner as

that employed for monocytes derived directly from animals (*i.e.* trypsinization, washing, and resuspension in the desired serum) and infected with virulent tubercle bacilli. The infected monocytes were cultivated in the Mackaness-type of chamber and followed in the usual fashion.

The results in Table VI reveal that *in vitro* cultivation of normal monocytes in immune serum failed to render these cells resistant to virulent tubercle bacilli; it may be noted that the per cent degeneration of these monocytes after 48 hours of incubation in the presence of immune serum following infection was 33, a value similar to that observed for normal monocytes (28 per cent) previously cultivated in normal serum medium and maintained in immune serum medium after infection. The per cent of infected monocytes in these two test systems was not markedly different and could not reasonably account for the absence of resistance in the test system consisting of normal monocytes previously cultivated in immune serum.

When immune monocytes were cultivated *in vitro* in normal serum medium for 24 hours and then infected with virulent tubercle bacilli and cultivated in immune serum medium, there was little degeneration (3 per cent) after 48 hours of incubation; the behavior of these cells resembled that of immune monocytes grown for 24 hours in immune serum medium before infection.

It is apparent that prior cultivation of normal monocytes in immune serum medium or of immune monocytes in normal serum medium failed to alter the inherent properties of these cells in so far as their resistance to necrotization by virulent tubercle bacilli was concerned.

Participation of Serum Factor during Parasitization and Cultivation.—In view of the preceding observations wherein immune serum failed to alter the capacity of virulent tubercle bacilli either to induce cellular degeneration or to proliferate intracellularly or to cause modification of the state of susceptibility of cells, it seemed essential to define further the conditions under which cellular resistance is manifested in the presence of immune serum. In particular, it was deemed necessary to determine the stage of tubercle bacillus–monocyte interaction at which participation of serum factors became critical; the two stages of bacilli-monocyte interaction studied in this section were parasitization (carried out at 4°C.) and post parasitization (incubation at 37°C.).

For the parasitization studies, immune monocytes were infected with virulent tubercle bacilli in the presence of fresh normal serum, fresh immune serum or heated (56°C. for 30 minutes) immune serum. In the post-parasitization experiments, the infected immune monocytes were cultivated in the presence of aged normal serum or aged immune serum.

Examination of the data given in the first two rows of Table VII reveals that parasitization of immune monocytes in presence of normal serum resulted in degeneration of the monocytes even though the infected immune monocytes were subsequently cultivated in the presence of immune serum medium; the

25 per cent degeneration noted for these cells after 48 hours of incubation equalled the degeneration observed for control monocytes (28 per cent) which were parasitized and subsequently cultivated in normal serum medium.

The necessity for serum factors during the post parasitization period of infected immune monocytes is shown in the central portion of Table VII. When immune monocytes were infected in the presence of fresh immune serum and cultivated in the presence of normal serum medium, degeneration occurred both at 24 and 48 hours (25 and 32 per cent respectively) as contrasted with the less than 5 per cent degeneration when similarly infected monocytes were cultivated in the presence of immune serum medium.

TABLE VII

Participation of Serum Factor during Parasitization and Cultivation

Type of monocyte*	Type of serum at parasitization	Type of serum during post parasitization	Per cent infected monocytes	Per cent degeneration‡ (hrs. after incubation)	
				24	48
Immune	Fresh normal serum§	Aged normal serum§	24	26	28
"	" " "	" immune "	24	20	25
"	" immune "§	" normal "§	22	25	32
"	" " "	" immune "	22	1	3
"	Heated " "§	" " "	20	4	5

* From tuberculin-positive rabbits immunized with BCG 30 or more days previously.
‡ Represents average per cent degeneration of monocytes in two or more culture chambers.
§ Fresh serum was obtained just prior to its use. Heated serum was inactivated at 56°C. for 30 minutes. Aged serum was kept in the refrigerator for 30 or more days.

It would appear from these experiments as well as other similar experiments (results not shown) that an important determinant of effective cellular resistance against virulent tubercle bacilli was continuous presence of immune serum.[1]

The fact that fresh immune serum was used during parasitization in the preceding experiments might appear to suggest a participation of complement or a complement-like substance during this stage of bacilli-monocyte interaction. This point was investigated by parasitization of immune monocytes in the presence of immune serum previously inactivated at 56°C. As may be seen in the last row of Table VII, immune monocytes infected in the presence of heated

[1] While not of immediate relevance to the present experiments, it may be of interest to note that attempts to analyze the effects of human sera from tuberculin-negative and tuberculin-positive individuals in protecting infected immune rabbit monocytes indicated that human serum was not compatible with rabbit monocytes and caused extensive destruction of these cells (results not shown).

immune serum successfully resisted necrotization by virulent tubercle bacilli (less than 5 per cent degeneration 48 hours after infection) if such infected immune monocytes were subsequently cultivated in the presence of immune serum.

Capacity of Intracellularly Passaged Bacilli to Cause Degeneration of Monocytes.—In consideration of the absence of activity of immune serum upon virulent tubercle bacilli and the observation of the preceding section wherein the effect of immune serum upon cellular resistance appeared to be a continuous one, it seemed reasonable to assume that modification of the bacterial cell, if it occurred at all, would most likely result from the combined action of immune cells and immune serum. This possibility was tested by studying the ability of intracellularly passaged bacilli to induce monocytic degeneration.

The bacteria used in the experiments shown in Table VIII consisted of a 6- to 7-day-old culture of the H37Rv strain of tubercle bacillus derived from (*a*) tween-albumin medium, (*b*) infected normal cells cultivated in normal serum medium, and (*c*) infected immune cells cultivated in immune serum medium. The tween-albumin cultures were prepared in the usual manner (6). The intracellularly passaged bacteria were prepared by parasitization of monocytes (normal or immune) with virulent bacilli, washing of the infected monocytes after parasitization to remove the bulk of unphagocytized bacteria, suspension of infected monocytes in serum (normal or immune) and cultivation of the infected cells in Carrel flasks (40,000 monocytes per flask) for 6 days at 37°C. At the end of this period the infected monocytes were lysed by addition of saponin to a final concentration of 2 per cent. The chemical was allowed to act for 1 hour at room temperature and lysis was facilitated by repeated pipetting of the samples. The bacteria liberated from the infected monocytes were then concentrated by centrifugation, washed several times in tween-albumin medium and finally resuspended in a small volume of the medium. When the bacterial suspension was found by microscopic examination to consist of mostly singly occurring bacilli, the number of bacilli was determined in a Petroff-Hausser chamber.

The various bacterial suspensions prepared as described were used to infect monocytes and cultures of the infected monocytes were prepared in the Mackaness-type of culture chamber and studied in the usual fashion.

There was no demonstrable difference in the capacity of the variously cultivated bacteria to cause degeneration of normal monocytes when these were infected and cultivated in the presence of normal serum medium. As may be seen in the first three rows of Table VIII, monocytes infected with *in vitro* grown bacteria or bacteria obtained from either a normal system (infected normal monocytes in normal serum medium) or an immune system (infected immune monocytes in immune serum medium) underwent degeneration as reflected in a 35 to 40 per cent drop in cell population 48 hours after infection. Similarly, when immune monocytes were infected with one or the other of these variously grown bacteria and cultivated in the presence of normal serum medium, degeneration of cells occurred; the decrease in monocyte population in this case ranged from 30 to 39 per cent (7th to 9th rows in Table VIII).

It is apparent from these results that intracellular passage of bacteria in a

normal or an immune system did not result in loss of the ability of virulent tubercle bacilli to cause destruction of normal and immune monocytes when the infected cells were maintained in normal serum medium.

Infection of normal monocytes with the various bacterial suspensions followed by cultivation of the infected monocytes in immune serum medium, however, revealed a difference in the capacity of these bacilli to induce degeneration of monocytes. Examination of Table VIII (rows 4 to 6) shows that *in vitro* grown bacteria and bacteria passaged in a normal system (normal cells and normal

TABLE VIII

Capacity of Intracellularly Passaged Bacilli to Cause Monocytic Degeneration

Type of monocyte	Source of bacilli for infection of monocytes	Cultivation serum for infected monocytes	Per cent infected monocytes	Per cent degeneration* (hrs. after incubation)
				48
Normal	Normal system‡	Normal	15	40
"	Immune "	"	16	40
"	Tween albumin	"	16	35
"	Normal system	Immune	18	37
"	Immune "	"	16	8
"	Tween albumin	"	15	42
Immune	Normal system	Normal	19	30
"	Immune "	"	17	32
"	Tween albumin	"	17	39
"	Normal system	Immune	15	3
"	Immune "	"	17	2
"	Tween albumin	"	19	0

* Represents average per cent degeneration in two or more culture chambers.

‡ Normal system indicates bacteria derived from infected normal monocytes cultivated in normal serum medium for 6 days at 37°C.; immune system refers to bacteria from infected immune monocytes cultivated in immune serum medium.

serum) caused considerable degeneration of monocytes (42 and 37 per cent respectively after 48 hours of incubation), but bacteria passaged in an immune system (immune monocytes and immune serum) exhibited a decreased potential for destruction of monocytes (8 per cent after 48 hours of incubation). While the 8 per cent loss in cell population in this experiment suggested partial retention of the bacterial capacity for destruction of monocytes, two other experiments (results not shown) have demonstrated that the bacteria recovered from an immune system caused no degeneration of monocytes after 48 hours of bacilli-monocyte interaction.

As might have been expected, infection of immune monocytes with any one

of the three bacterial preparations followed by cultivation of the infected cells in immune serum medium resulted in no loss in cell numbers after 48 hours of bacilli-monocyte interaction (last 3 rows of Table VIII).

It would appear that under appropriate conditions of testing, namely infection of normal monocytes followed by cultivation of infected cells in immune serum medium, variations in the ability of various suspensions of the same strain of tubercle bacillus to cause degeneration of monocytes was demonstrable, and it would seem implicit in these observations that these varying capacities for destruction of monocytes were a reflection of the past history of the microbe.

TABLE IX

Multiplication of Bacilli in Normal and Immune Systems and in Cultivation Media

Type of monocyte infected	Cultivation serum for: infected monocytes or bacteria	Per cent infected monocytes	No. bacteria per infected monocyte	Bacterial-fold increase* (hrs. after incubation)		
				48	96	144
Normal	Normal	17	5	2.2	9.5	16.2
Immune	Immune	18	4	2.2	10.3	18.5
None	Normal‡	0	0	0.8	1.0	0.7
"	Immune‡	0	0	1.0	0.8	0.7

* The infected monocytes were cultured in Carrel flasks with 40,000 monocytes added to each flask. The infected cells were lysed after the designated period of incubation (3 Carrel flasks from each sample at each interval) and the entire contents of the flasks used for determination of viable bacteria. The values given represent the average value of two separate experiments.

‡ The cultivation medium consisting of 40 per cent serum in Tyrode's solution was seeded with 1×10^5 bacteria per ml. and incubated at 37°C. Samples were removed for viability determinations at the designated intervals.

The observed difference in ability of these bacteria to cause monocytic degeneration was not attributable to major differences in the numbers of infected monocytes since these ranged from 15 to 19 per cent of the total cell population.

It is conceivable that the variation in ability of the bacteria to destroy monocytes may in part result from deviations in the numbers of viable bacteria present in the various bacterial suspensions which were used for parasitization of the normal monocytes shown in Table VIII. This was tested, as previously described, by pooling the entire contents of three Carrel flasks at selected intervals of time and determining on glycerol blood agar plates the numbers of viable bacteria.

The results of viability tests, as shown in Table IX, indicate very little difference in the viability of bacteria derived from infected normal monocytes cultivated in normal serum medium and those extracted from infected immune

monocytes maintained in immune serum medium. The bacterial-fold increases at 48, 96, and 144 hours after infection were quite similar in both tissue culture systems.

The results of seeding normal serum medium and immune serum medium with 1×10^5 virulent tubercle bacilli and determining the rate of growth of the bacteria in these media indicated that the bacilli failed to increase in numbers over a period of 6 days. This observation would seem to suggest that the bacteria grown in either a normal or an immune system consisted predominantly of intracellularly propagated bacteria.

DISCUSSION

While there is no definitive information on the mechanism of resistance to tuberculosis, certain observations (6, 7, 11, 12) have suggested that it may be partially dependent upon cellular and humoral factors. At the "tissue culture level" it was found that immune monocytes from BCG-immunized animals could be protected against the necrotizing action of virulent tubercle bacilli by introduction of immune sera derived from animals immunized with either tubercle bacilli or agents totally unrelated to *Mycobacterium* (7). As reported herein, the seemingly non-specific activity of immune sera was further substantiated by the observations that globulin derived from immune sera failed to protect immune monocytes against virulent bacilli and absorption of immune sera with homologous or closely related antigens did not remove the protective serum factor.

The absence of correlation between hypersensitivity and resistance in the intact animal has been reported repeatedly and was reaffirmed in several recent publications (13–15). The findings presented in this paper indicated that resistance at the cellular level was also dissociable from the hypersensitive state, as evidenced by the resistance of monocytes in the absence of a positive skin reaction.

Although earlier observations have shown that resistance against tubercle bacilli required both immune cells and immune serum, it was not established whether the two components appeared simultaneously. The present experiments indicated that early elicitation of protective serum factor was possible with heat-killed bacilli, but early induction of cellular resistance required injections of viable tubercle bacilli. It may be of interest to note that the constituents of heat-killed *Mycobacterium* have been shown to affect the resistance of animals in other ways (16, 17).

Studies aimed at elucidation of the mode of action of immune sera revealed an absence of direct effect of serum upon tubercle bacilli; thus, preliminary *in vitro* treatment of virulent tubercle bacilli with immune serum failed to impair their capacity either to cause monocytic degeneration or to multiply intracellularly. The possibility that immune serum may act indirectly by alteration of

cellular susceptibility was also investigated, and it was found that the inherent susceptibility of normal monocytes to virulent tubercle bacilli could not be altered by prolonged contact with immune serum. Since these studies revealed that prolonged and continuous contact of immune monocytes with immune serum was essential for resistance against the necrotizing action of virulent bacilli, it was deemed desirable to investigate the properties of intracellularly passaged bacteria.

The present paper has reported that the capacity of virulent tubercle bacilli to cause degeneration of monocytes may be impaired by passage of virulent bacilli in an immune system (immune monocytes cultivated in immune serum). Intracellular passage alone, however, was not responsible for this alteration of the bacterial capacity for cell destruction, since bacteria similarly passaged in normal monocytes followed by cultivation of infected cells in normal serum medium failed to effect this transformation. The nature of the bacterial change has not been established in these studies, but it seems pertinent to note that the altered capacity of virulent bacilli to cause destruction of normal monocytes was demonstrable only in the presence of immune serum. A possible interpretation would be that modification of the bacterial cell surface resulting from intracellular passage in an immune system rendered the bacterial cell more sensitive to the combined action of immune serum and monocyte enzyme systems, thus allowing for effective disposal of the sensitized bacilli by components primarily cellular in origin.

SUMMARY

Studies of the relationship of protective serum factor to cellular resistance and to tuberculin skin sensitivity have demonstrated that protective serum factor may exist independently of a high level of cellular resistance, and that both protective factor and cellular resistance may be demonstrable without a concomitant hypersensitivity of the delayed type.

The experiments with absorbed sera and the globulin fraction of immune serum indicated no specific association of protective serum factor with antibody globulin.

The protective factor in immune serum was found to be thermostable and non-dialyzable.

In vitro exposure of virulent tubercle bacilli to the immune serum from BCG-immunized animals failed to alter the bacterial capacity for destruction of monocytes and for intracellular proliferation.

In vitro cultivation of normal and immune monocytes in normal or immune serum was not effective in changing the native susceptibility or resistance of these cells.

Effective manifestation of resistance to virulent tubercle bacilli by immune monocytes was found to require the continuous presence of immune serum.

The intracellular passage of virulent tubercle bacilli in an immune system (immune monocytes cultivated in immune serum) resulted in a decreased bacterial potential for destruction of normal monocytes when these were cultivated in the presence of immune serum.

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