STUDIES ON TUBERCLE BACILLUS-MONOCYTE RELATIONSHIP*

II. Induction of Monocyte Degeneration by Bacteria and Culture Filtrate: Specificity of Serum and Monocyte Effects on Resistance to Degeneration

By JACOB FONG, Ph.D., PATRICIA SCHNEIDER, and SANFORD S. ELBERG, Ph.D.

With the Technical Assistance of James Akiyama

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

(Received for publication, September 12, 1956)

Despite intensive research on tuberculosis and the tubercle bacillus, the important and basic problems of virulence and resistance remain a challenge.

Studies of acquired immunity have revolved around the relative importance of humoral and cellular factors. As previously indicated (1), valid conclusions concerning the role of cells in acquired immunity against tuberculosis require analyses of bacterium-cell relationships during periods when the *in vitro* cell populations show no change in cell numbers. Under such conditions, it was observed that the virulent H37Rv strain of tubercle bacillus regularly induced degeneration of both normal and immune monocytes (derived from rabbits immunized with BCG) when these were cultivated *in vitro* in the presence of normal rabbit serum (derived from tuberculin-negative rabbits); in contrast, the serum of rabbits immunized with BCG protected immune but not normal cells against the degenerative effects of virulent tubercle bacilli. The first of these observations posed a possible relationship between virulence and the factor in tubercle bacilli which promoted degeneration of cells cultivated *in vitro*. The protection of immune monocytes by the serum of rabbits immunized with BCG suggested an hitherto unexplored role for serum components in resistance against tuberculosis.

The present paper represents an investigation along the lines suggested by these earlier observations. It describes the different abilities of virulent, attenuated, and avirulent strains of tubercle bacilli to induce degeneration of monocytes cultivated *in vitro*. It also describes the activity of bacterial culture filtrates in promoting cell degeneration and compares this activity of bacterial filtrates with those of purified protein derivative and old tuberculin. Finally, it presents data which indicate that an element of specificity is involved in the resistance of monocytes against virulent tubercle bacilli and further, that such resistance is mediated by serum components which are seemingly non-specific in nature.

* This work was supported by a grant from the Alameda County (California) Tuberculosis Association, and by ONR Grant Nonr-645(00).

Materials and Methods

Most of the materials and methods used in these experiments were described in detail in a previous report (1), and are therefore considered briefly in this paper. Complete details are given for materials and methods not previously described.

Monocytes.—Normal adult rabbits, rabbits immunized with BCG, or rabbits immunized with *Salmonella rutgers* were used as monocyte donors. The monocytes were obtained by washing the peritoneal cavity of rabbits with 300 to 400 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 minutes, and the sedimented cells were thoroughly redispersed in 6 to 8 ml. of freshly prepared 0.25 per cent trypsin in Tyrode's solution. After 25 minutes at room temperature (24 to 26°C.), the cells were washed several times with large volumes of Tyrode's solution; the packed cells from the last washing were resuspended in a few milliliters of freshly collected serum (normal, anti-BCG or anti-*Salmonella*) and the number of cells determined in a hemocytometer. These cells were parasitized with bacteria or exposed to bacterial products in the manner described below.

Bacteria.—The strains of *Mycobacterium tuberculosis* used in these studies were H37Rv, H37Ra, and BCG, all of which were originally obtained from the Phipps Institute, Philadelphia.

The two human strains of tubercle bacilli were maintained on Trudeau medium. For use in parasitization of monocytes, transplants of the stock culture were made in tween-albumin liquid medium and incubated 7 days at 37°C. The week-old culture of bacteria 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 large aggregates. The supernatant fluid, which consisted largely of bacteria occurring singly, was used for parasitization of monocytes after determination of bacterial cell numbers in a Petroff-Hausser chamber under dark-ground illumination.

The BCG strain of *Mycobacterium tuberculosis* was maintained on Calmette's potato medium. The procedure for preparing BCG cultures for use in parasitization of monocytes was identical with that employed in preparation of human strains of bacilli. In preparing cultures of BCG for immunization of rabbits, the bacterial growth from Calmette's potato medium was ground with steel balls, suspended in physiological saline and standardized to contain 2.5 to 5.0 $\times$ 10^8 bacteria per ml.

The strain of *Salmonella rutgers* 495 was obtained from the Division of Laboratories, California State Department of Health, Berkeley. In preparing antigens for immunization of rabbits, transplants of *Salmonella rutgers* were made in large flat bottles containing nutrient agar; these were incubated for 18 to 24 hours at 37°C. The bacterial growth on the surface of the agar was washed off with a small volume of physiological saline, and an equal volume of 0.6 per cent formalin was added. The formalinized suspension was kept at 37°C. until complete inactivation of the bacteria occurred. The inactivated suspension was adjusted with formalinized saline to contain 9.0 $\times$ 10^9 bacteria per ml. and used for immunization of rabbits.

Culture Filtrate.—A week-old tween-albumin culture of tubercle bacilli was passed through an ultrafine sintered glass filter; the clear, cell-free filtrate was used without further treatment.

Old Tuberculin.—This was a 4 times concentrated preparation of old tuberculin obtained from the Lederle Laboratories, American Cyanamid Co., Pearl River, New York. The O.T. was diluted 1:120 with sterile physiological saline before use.

Purified Protein Derivative.—A 50 μg. P.P.D. tablet (Parke, Davis and Co., Detroit) was dissolved in 1 ml. of sterile physiological saline for use in these experiments.

Parasitization of Monocytes.—The desired strain of tubercle bacillus was mixed with the appropriate suspension of monocytes (monocytes of normal rabbits, rabbits immunized with BCG, or rabbits immunized with *Salmonella rutgers* and suspended in normal, anti-BCG,

anti-*Salmonella*, or anti-ovalbumin serum) 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 for 1 hour at 4°C. At the end of this time the supernatant fluid was discarded and the sedimented cells resuspended in a small volume of the appropriate nutrient medium (see section on Nutrient Media). The number of monocytes was determined in a hemocytometer, and the infected monocyte suspension was diluted with sufficient amounts of the proper nutrient medium to yield approximately 15 monocytes per c.mm.; the diluted suspension of infected monocytes was used for *in vitro* cultures as described below.

Exposure of Monocytes to Bacterial Products.—When a bacterial culture filtrate was used instead of bacteria, 0.1 ml. of the culture filtrate was mixed with 0.4 ml. of the desired monocyte suspension (adjusted to contain 2.5×10^6 monocytes per ml.). The mixture was kept for 10 minutes at room temperature and for 1 hour at 4°C. At the end of this time the sample was diluted with the desired nutrient medium to yield approximately 15 cells per c.mm. and the diluted sample used for *in vitro* cultures.

Exposure of monocytes to O.T. or P.P.D. consisted of mixing 0.1 ml. of the reagent (1:120 O.T., solution of P.P.D. containing 50 μg. per ml.) with 0.4 ml. of monocyte suspension containing 2.5×10^6 monocytes per ml. Further treatment of these samples followed the procedure outlined above for monocytes exposed to culture filtrate.

Control suspensions of monocytes were handled in a manner identical with that of infected monocytes except that an appropriate volume of tween-albumin medium was added instead of bacteria.

Nutrient Media.—The media used for cultivation of monocytes consisted of 40 per cent rabbit serum (normal, anti-BCG, anti-*Salmonella* or anti-ovalbumin) in Tyrode's solution. The particular type of serum used in an experiment will be indicated in the table which summarizes the results of the experiment. The pH of the medium was adjusted to 7.4 with 5 per cent CO_2 in air.

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

Anti-BCG serum consisted of the pooled sera of tuberculin-positive rabbits. The animals were given 1 to 3 injections of BCG (0.2 to 0.3 ml. intradermally in the first dose and similar amounts intravenously in subsequent doses with about 30 days intervening between injections) and bled 4 to 5 weeks after the last injection.

Anti-*Salmonella* serum represented the pooled sera of rabbits immunized with *Salmonella rutgers*. Immunization consisted of 6 injections of *Salmonella* antigen administered on alternate days. The first dose was 0.5 ml. of antigen inoculated subcutaneously. All subsequent injections were made with equal volumes of antigen inoculated both subcutaneously and intravenously. The volume of antigen was doubled each time until a maximum of 3.0 ml. was administered by each of the two routes. The rabbits were bled 1 week after the last injection. The agglutinating titer of this serum, as determined by titration against the flagellar antigens of *Salmonella rutgers*, exceeded 2560 (reciprocal of the highest dilution of serum still exhibiting observable agglutination).

Anti-ovalbumin serum was obtained from rabbits immunized with an egg albumin solution. This solution was prepared by dissolving 630 mg. of crystalline egg albumin in 600 ml. of saline. After sterilization by filtration through a Seitz filter, 0.3 ml. of a sterile solution of 10 per cent $K_2Al_2SO_4$ was added. This was followed by the addition of 1 N NaOH until maximum precipitation of the alum occurred. Immunization consisted of 4 daily injections of 1.0 to 2.0 ml. of egg albumin solution administered intraperitoneally or intravenously. After a 3 day rest period, 5 daily injections of 2.0 to 3.0 ml. of egg albumin solution were given intravenously. The antibody nitrogen of the serum 1 week after the last injection of antigen averaged 0.105 mg. N/ml. The anti-ovalbumin serum used in these experiments was a sample of serum which had been stored in a CO_2 cabinet for some time.

Cultivation of Monocytes.—This was carried out in the culture chambers described by Mackaness (2). Approximately 0.05 ml. of diluted monocyte suspension (prepared as described under parasitization of monocytes) 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 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. Quantitative enumeration of monocytes with variations of 5 per cent or less was made possible by a special square constructed in the eyepiece of the microscope and by calibrated stage markings which were correlated with the square in the eyepiece. Full details of this were described in a previous paper (1).

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; a small amount of the parasitized cell suspension was introduced into the space confined by the plastic ring. After adherence of cells to the coverslip, the fluid was removed with a capillary pipette 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.—The percentage of infected monocytes after parasitization 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 (monocytes with more than 20 bacteria were counted as having 20) and dividing this total by the number of infected monocytes.

EXPERIMENTAL

Effect of Various Strains of Tubercle Bacilli upon Monocytes.—Various investigators (3–7) have observed that attenuated strains of tubercle bacilli such as RlRv and BCG were able to multiply intracellularly in tissue cultures and in laboratory animals whereas an avirulent strain such as H37Ra was unable to do so. Since a virulent strain (H37Rv) of tubercle bacillus has been shown to induce degeneration of monocytes (as evidenced by a reduction in cell numbers and by the appearance of non-refractile cells with indefinite cell boundaries and little evidence of internal structure) cultivated *in vitro* in the presence of normal serum (1), it seemed of interest to compare the relative ability of virulent, attenuated, and avirulent strains of tubercle bacilli to promote degeneration of normal monocytes.

A representative experiment of this type is shown in Table I. The monocytes of tuberculin-negative rabbits were infected with H37Rv, BCG, or H37Ra and cultivated in the presence of normal serum. It is evident that the three strains of tubercle bacilli differed in their ability to effect degeneration of monocytes. Marked degeneration of monocytes infected with the virulent H37Rv strain was apparent after only 10 hours of incubation; by 48

hours, almost one-half of the original cell population had been lost. Induction of monocytic degeneration by the attenuated BCG strain of tubercle bacillus was apparent at the 24 hour interval, but the degree of degeneration was slight. The amount of monocytic degeneration doubled during the next 24 hours of incubation, but it was still considerably less than that caused by the H37Rv strain. Infection of monocytes with the avirulent H37Ra strain resulted in no apparent effect upon the cells during the entire period of observation.

The percentage of infected monocytes and the average number of bacteria per infected monocyte are shown in Table I. The average number of bacteria per infected monocyte was lowest for monocytes parasitized with the H37Ra

TABLE I

Survival of Cells after Infection with Various Strains of Tubercle Bacilli

Treatment of cells*	Per cent infected monocytes‡	Average No. bacteria per infected monocyte	Average per cent degeneration§ (hrs. after incubation)		
			10	24	48
Uninfected controls	0	0	0	0	0
Infected with H37Rv	15	7.3	31	33	41
" " BCG	12	8.4	0	12	24
" " H37Ra	14	6.4	0	0	0

* Monocytes were derived from normal (tuberculin-negative) rabbits; ratio of bacteria to monocyte was 10:1 for all three strains of tubercle bacilli. Monocytes were cultivated in presence of normal rabbit serum.

‡ Obtained by examining 200 stained cells.

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

strain, but the difference between this strain and the H37Rv strain was no greater than that between the H37Rv and the BCG strains; such differences would certainly not account for the different behavior of these three strains of tubercle bacilli.

Effect of Bacterial Products upon Monocytes.—The relationship between tuberculin hypersensitivity and white blood cell types has been demonstrated by a number of workers (8–11), and Favour (12) has described the cytotoxic effect of tuberculin on fluid suspensions of white blood cells of tuberculous animals. For reasons indicated previously (1), it was believed that induction of cell degeneration by virulent tubercle bacilli bore little relationship to the cytotoxicity of tuberculin. This conclusion would seem to be substantiated by the results of Table II. The complete procedural details of these experiments are given in the section on Materials and Methods. Basically these experiments tested the ability of normal and immune cells suspended in normal or immune serum (from non-immunized animals and animals immunized with

TABLE II

Effect of H37Rv, O.T., P.P.D., and Culture Filtrate upon in Vitro Survival of Monocytes

Type of monocyte*	Monocytes exposed to‡	Type of serum§	Average per cent degeneration‖ (hrs. after incubation)			
			4	10	20	44
Normal	Tween-albumin	Normal	0	1	1	X
"	"	Immune	0	1	3	X
Immune	"	Normal	4	4	4	X
"	"	Immune	4	4	3	X
Normal	H37Rv	Normal	1	20	27	47
"	"	Immune	0	1	13	21
Immune	"	Normal	0	5	20	38
"	"	Immune	2	2	2	5
Normal	O.T.	Normal	10	10	10	X
"	"	Immune	11	11	7	X
Immune	"	Normal	24	26	23	24
"	"	Immune	24	24	23	X
Normal	P.P.D.	Normal	—	—	0	—
"	"	Immune	—	—	1	—
Immune	"	Normal	—	—	37	—
"	"	Immune	—	—	27	—
Normal	Culture filtrate	Normal	—	—	31	—
"	" "	Immune	—	—	7	—
Immune	" "	Normal	—	—	32	—
"	" "	Immune	—	—	0	—

* Normal designates monocytes of tuberculin-negative rabbits; immune refers to monocytes of rabbits rendered tuberculin-positive by BCG immunization.

‡ Ratio of H37Rv to monocyte was 10:1; O.T. (4 times concentrated) was diluted 1:120 before use; the solution of P.P.D. contained 50 μg. per ml.; the culture filtrate was from a week-old culture of H37Rv in tween-albumin medium. The method of exposing monocytes to O.T., P.P.D., or culture filtrate was described in the section on Materials and Methods.

§ Refers to the type of serum (normal or anti-BCG) in which monocytes were suspended at time of parasitization or exposure to O.T., P.P.D., or culture filtrate. The same type of serum was used for subsequent cultivation of monocytes.

‖ Refers to average per cent degeneration (from original count of approximately 500 to 1000 monocytes) of 2 to 3 cultures. X, indicates increase in number of monocytes; —, indicates sample not counted.

BCG respectively) to survive treatment with H37Rv, O.T., P.P.D., and a filtrate of a week-old culture of H37Rv in tween-albumin medium.

Comparison of the results following exposure of monocytes to H37Rv with those recorded for O.T. reveals a number of interesting differences. Normal monocytes cultivated in the presence of normal serum were affected by ex-

posure to both agents. As opposed to the results obtained with H37Rv, however, the degeneration of monocytes caused by O.T. appeared sooner (4 hours), was less pronounced (10 per cent as against 20 to 47 per cent for H37Rv), and showed no progression with time; these same differences between the effects of O.T. and H37Rv may be observed both for normal cells cultivated in the presence of immune serum and for immune cells in normal serum. It is apparent, however, that immune cells were more susceptible to the effects of O.T. than were normal cells (24 per cent as against 10 per cent for normal cells). The most significant difference between the effects of O.T. and H37Rv was exhibited by immune cells cultivated in the presence of immune serum; under these conditions, the immune cell was protected against the degenerative effects of H37Rv (the average per cent degeneration of 5 shown for the 44 hour interval was within the limits of error in the method of counting monocytes), but not against the cytotoxic action of O.T. (as evidenced by the early occurrence and the high percentage of degenerated cells). It may also be of some interest to note that 3 of the 4 cultures treated with O.T. showed multiplication of cells after 44 hours of incubation whereas cell proliferation had not yet commenced at this time in any of the cultures infected with H37Rv.

The effects of P.P.D. upon normal and immune monocytes cultivated in the presence of normal or immune serum are shown for the 20 hour period. A comparison of these results with those recorded for H37Rv and O.T. at the same time interval discloses a similarity in action of P.P.D. and O.T. It may be observed that both substances caused marked degeneration of immune cells, and that the action of both, unlike that of H37Rv, was unaffected by the type of serum used. Since P.P.D. exhibited little cytotoxic effect on normal cells, it would seem that the low level of cell degeneration evoked by O.T. in the cultures of normal monocytes may be referable to certain impurities in the latter reagent.

When the results of tests on a culture filtrate of H37Rv are compared with those given by the viable intact bacterial cell, a definite parallelism in action becomes apparent. Thus, both agents caused marked degeneration of normal and immune monocytes when these were cultivated in the presence of normal serum. Similarly, cultivation of immune cells in an immune serum resulted in protection of these monocytes against the degenerative effects of both agents. Although the potency of culture filtrates may vary from one experiment to another, the basic similarity in action of H37Rv and its culture filtrate has been confirmed in other experiments (results not shown).

It would appear on the basis of these findings that the effects of H37Rv upon monocytes differed from that of O.T. and P.P.D. but were indistinguishable from that of a culture filtrate of the virulent bacilli.

Non-specificity of Serum Action in Protection of Monocytes against Virulent Tubercle Bacilli.—A previous report (1) has shown that the serum of rabbits

immunized with BCG delayed the bacilli-induced degeneration of normal monocytes and prevented its occurrence in cultures of immune monocytes. The specificity or non-specificity of the humoral components in the serum of rabbits immunized with BCG was not established in this earlier investigation. One of a number of similar experiments designed to establish the nature of this serum activity is shown in Table III. The results in this table compare the survival of normal and immune monocytes infected with virulent H37Rv and

TABLE III

Effect of Various Sera upon Survival of Monocytes Infected with H37Rv

Type of monocyte*	Type of serum‡	Per cent of infected cells§	Average per cent degeneration‖ (hrs. after incubation)	
			10	24
Normal	Normal	25	36	38
"	Anti-BCG	22	2	1
"	Anti-*Salmonella*	19	0	4
"	Anti-ovalbumin	—	—	0
Immune	Normal	26	2	11
"	Anti-BCG	19	0	1
"	Anti-*Salmonella*	20	0	2
"	Anti-ovalbumin	—	—	0

Control (uninfected) monocytes suspended in the different types of serum showed no degeneration.

—, indicates sample not counted.

* Normal refers to monocytes from tuberculin-negative rabbits; immune refers to monocytes from rabbits vaccinated with BCG.

‡ Normal designates serum of tuberculin-negative rabbit; anti-BCG refers to serum of rabbit immunized with BCG; anti-*Salmonella* refers to serum of rabbit immunized with *Salmonella rutgers*; anti-ovalbumin refers to serum of rabbit immunized with a solution of crystalline egg albumin.

§ Obtained by examination of 200 stained cells.

‖ Average per cent degeneration (from initial count) of 2 to 3 cultures.

cultivated in the presence of normal serum, homologous antiserum, or heterologous antiserum.

Examination of the data in the upper half of Table III reveals a protection of normal monocytes by homologous antiserum (from rabbits immunized with BCG) and by heterologous antiserum (serum of rabbits immunized with *Salmonella rutgers* or with a solution of crystalline egg albumin); this was evidenced by an absence of monocyte degeneration as reflected in a constant average per cent degeneration over a 24 hour period. No such protection of normal monocytes was evident with normal rabbit serum (average per cent degeneration of 38 over the 24 hour period).

As might have been expected from the results obtained for normal mono-

cytes, it may be seen that a similar protection was afforded immune cells by either homologous or heterologous antisera but not by normal serum (no degeneration in immune sera as compared with 11 per cent in normal serum after 24 hours).

There was very little difference between the behavior of normal and immune monocytes over the 24 hour period of observation in this experiment. Various

TABLE IV

Specificity of Monocytes in Resistance to Infection with H37Rv

Source of monocyte*	Type of serum‡	Per cent infected monocytes§	Average per cent degeneration‖ (hrs. after incubation)		
			10	24	48
Normal	Normal	9.5	35	39	50
"	Anti-BCG	16.5	1	20	49
Immune	Normal	10.0	0	23	41
"	Anti-BCG	15.0	0	1	3
Salmonella	Normal	16.5	30	43	46
"	Anti-BCG	17.5	—	18	42
Normal	Normal	16.5	14	25	37
Immune	Anti-BCG	14.0	0	0	0
Salmonella	Anti-*Salmonella*	29.5	0	27	35

The different types of control (uninfected) monocytes suspended in the various sera showed no change in cell numbers during the experimental period.

—, indicates sample not counted.

* Monocytes derived from normal (tuberculin-negative) and immune (tuberculin-positive) rabbits and from rabbits immunized against *Salmonella rutgers*.

‡ Normal refers to serum of tuberculin-negative rabbits; anti-BCG refers to serum of rabbits immunized with BCG; anti-*Salmonella* refers to serum of rabbits immunized with *Salmonella rutgers*.

§ Obtained by examination of 200 stained cells. Ratio of H37Rv to monocyte was 10:1.

‖ Average per cent degeneration (from initial count) of 2 to 3 cultures.

other experiments (1) have demonstrated that immune (anti-BCG) serum only delayed the onset of degeneration in infected normal monocyte cultures. Whether or not anti-ovalbumin and anti-*Salmonella* sera behaved identically in this respect was not established in these experiments.

It may be observed that there are minor differences in the per cent of infected monocytes shown in Table III. These minor differences would scarcely account for the lack of protection of monocytes cultivated in normal serum.

Specificity of Monocytes in Resistance to H37Rv.—The results presented in Table III have revealed an element of non-specificity in the action of serum components, for immune sera against *Salmonella rutgers* and against ovalbumin proved equally effective in protecting immune monocytes against the

necrotizing action of virulent tubercle bacilli. It seemed necessary, therefore, to examine the monocytes from this view-point. The results of one of a number of similar experiments designed to test the specificity of monocytes in resistance against tubercle bacilli are presented in Table IV.

The three types of monocytes which were studied were derived from normal (tuberculin-negative) rabbits, immune rabbits (immunized with BCG and rendered tuberculin-positive) and rabbits immunized with a formalinized suspension of *Salmonella rutgers*. These monocytes were infected with H37Rv (10 bacilli per monocyte) and cultivated either in normal serum or in immune serum obtained from tuberculin-positive rabbits.

The results shown in the upper half of Table IV indicate a similarity in the behavior of normal monocytes and monocytes derived from an animal immunized with *Salmonella rutgers*. Thus, both normal monocytes and the monocytes of *Salmonella*-immunized rabbits showed early (10 hours) and marked degeneration of monocytes (30 to 35 per cent in 10 hours and 46 to 50 per cent in 48 hours) when these were infected and cultivated in normal serum; moreover, the degeneration of both types of monocytes was delayed (a lower level of monocytic degeneration in 24 hours as opposed to the marked degeneration usually encountered when normal serum was used) but not suppressed by immune (anti-BCG) serum. It is in connection with the cultivation of monocytes in immune serum that the monocytes of the normal animal and the animal immunized with *Salmonella rutgers* differed sharply from the truly immune monocyte (derived from an animal immunized with BCG); the immune monocyte maintained in the presence of immune serum proved completely refractory to the degenerative effects of virulent tubercle bacilli.

It might be reasoned that the monocytes of an animal immunized with *Salmonella rutgers* would exhibit its full potentialities only when suspended in homologous (anti-*Salmonella*) antiserum. This was tested and the results are shown in the lower part of Table IV. It is evident that after infection with H37Rv, cultivation of the monocytes of an animal immunized with *Salmonella rutgers* in homologous antiserum failed to confer on such cells a state of resistance equivalent to that of the truly immune monocyte. The behavior of the monocyte of the *Salmonella*-immunized rabbit in anti-*Salmonella* serum resembled its behavior when cultivated in the presence of heterologous (anti-BCG) antiserum (a delayed degeneration which appeared at the 24 hour period and not at the 10 hour interval).

DISCUSSION

The constituents of tubercle bacilli which act as determinants of virulence have been studied by various investigators.

Choucroun (13–16) has described the toxicity of a lipopolysaccharide (PMKO) and discussed its possible role in virulence and immunity. Bloch (17, 18) has reported

on the ability of "cord factor" to inhibit the migration of leucocytes in tissue cultures and on its lethality for mice. Spitznagel and Dubos (19) have shown that a toxic fraction soluble in monochlorobenzene and insoluble in cold petroleum ether may be extracted from tubercle bacilli. The precise role of these various constituents is by no means clear, and as Dubos (20) has indicated, the cells of the avirulent strain H37Ra may have a primary toxicity as high as those of BCG, RlRv, or of the human and bovine virulent strains.

It remains to determine whether such toxins are ecologically significant. The ability of virulent but not of avirulent tubercle bacilli to induce degeneration of normal monocytes cultivated *in vitro*, as described herein, is of immediate relevancy to the whole problem of virulence of tubercle bacilli. These observations may be interpreted in terms of a difference in the amount and/or distribution of a virulence component (the degeneration-promoting factor). On the other hand, it is perhaps even more reasonable to assume a possible difference in the ability of the three strains of tubercle bacilli to elaborate this substance (and by inference also a difference in the ability of the three strains to survive) under the present conditions of experimentation, particularly in view of the obvious parallelism in action of H37Rv and its filtrate.

The role of allergy in the pathogenesis of tuberculosis is generally accepted, and the cytotoxicity of tuberculin is well authenticated (8–12). The induction of monocytic degeneration by virulent tubercle bacilli could conceivably have a similar underlying mechanism. An analysis of this possibility has been carried out, and the results have indicated an independence of the two observations. A basic difference between the two phenomena was concerned with the action of these substances upon immune cells derived from an animal immunized with BCG. It was observed that O.T. and P.P.D. had their greatest effect upon these immune cells and that their activity was not affected by antiserum; in contrast, normal monocytes were most severely affected by virulent tubercle bacilli and immune monocytes were completely protected against the virulent H37Rv when immune serum was present. The role of monocytic degeneration in the pathogenesis of tuberculosis is unclear at this time but is worthy of consideration for future studies.

Investigations on the role of specific antibodies in immunity against tuberculosis have yielded evidence of a negative nature (21–25). The ability of immune (anti-BCG) serum to prevent induction of degeneration in cultures of infected immune cells (monocytes of rabbits immunized with BCG) implied a participation of either specific antibody or non-specific serum components in resistance against virulent tubercle bacilli. Since immune sera against such diverse antigens as those of *Salmonella rutgers* and ovalbumin protected immune cells against virulent H37Rv, it would appear to establish the importance of non-specific serum factors. At the same time, the inability of normal serum to afford a similar protection suggested that these serum components which

mediated the resistance of monocytes against virulent tubercle bacilli were either found only in immune sera or were present in effective concentrations only in such sera.

The present findings have demonstrated that the resistance of monocytes against the degenerative effects of H37Rv may be of a specific nature. This was indicated by the observation that monocytes of rabbits immunized with BCG and cultivated in the presence of immune serum (anti-BCG or anti-*Salmonella*) withstood the effects of parasitization with H37Rv. No such resistance was noted for monocytes derived from animals immunized with *Salmonella rutgers*, despite the presence of immune serum.

SUMMARY

Studies of virulent, attenuated, and avirulent strains of tubercle bacilli have demonstrated the proficiency of virulent strains to effect degeneration of normal monocytes cultivated in the presence of normal serum. Attenuated strains were less active in this respect, and avirulent bacilli failed to induce monocytic degeneration.

Comparison of the effects of virulent H37Rv with O.T., P.P.D., and a culture filtrate of H37Rv revealed a similarity in action of H37Rv and its filtrate. The action of O.T. and P.P.D. differed from that of H37Rv in that the greatest effect of H37Rv was upon normal cells as opposed to the effect of O.T. and P.P.D. upon immune cells. Additionally, it was demonstrated that immune serum (anti-BCG) protected immune cells against H37Rv but not against O.T. or P.P.D.

The protection of immune cells by heterologous antisera (anti-*Salmonella* and anti-ovalbumin) as well as by homologous antiserum (anti-BCG) against the degenerative effects of H37Rv indicated a non-specificity in action of serum factors. The ability of the monocytes of animals immunized with BCG and the failure of monocytes of animals immunized with *Salmonella rutgers* to withstand parasitization with H37Rv, when both types of monocytes were cultivated in immune (anti-BCG) serum, indicated a specificity of cellular resistance.

BIBLIOGRAPHY

1. Fong, J., Schneider, P., and Elberg, S. S., Studies on tubercle bacillus-monocyte relationship. I. Quantitative analysis of effect of serum of animals vaccinated with BCG upon bacterium-monocyte system, *J. Exp. Med.*, 1956, **104**, 455.
2. Mackaness, G. B., The action of drugs on intracellular tubercle bacilli. *J. Path. and Bact.*, 1952, **64**, 429.
3. Steeken, W., and Gardner, L. U., History of H37 strain of tubercle bacillus, *Am. Rev. Tuberc.*, 1946, **54**, 62.
4. Pierce, C. H., Dubos, R. J., and Middlebrook, G., Infection of mice with mammalian tubercle bacilli grown in tween-albumin liquid medium, *J. Exp. Med.*, 1947, **86**, 159.

5. Pierce, C. H., Dubos, R. J., and Schaefer, W. B., Multiplication and survival of tubercle bacilli in the organs of mice, *J. Exp. Med.*, 1953, **97**, 189.
6. Suter, E., The multiplication of tubercle bacilli within normal phagocytes in tissue culture, *J. Exp. Med.*, 1952, **96**, 137.
7. Mackaness, G. B., Smith, N., and Wells, A. Q., The growth of intracellular tubercle bacilli in relation to their virulence, *Am. Rev. Tuberc.*, 1954, **69**, 479.
8. Rich, A. R., and Lewis, M. R., The nature of allergy in tuberculosis as revealed by tissue culture studies, *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.
9. Aronson, J. D., The specific cytotoxic action of tuberculin in tissue culture, *J. Exp. Med.*, 1931, **54**, 387.
10. Heilman, D. H., Feldman, W. H., and Mann, F. C., Specific cytotoxic action of tuberculin: Quantitative studies on tissue culture, *Am. Rev. Tuberc.*, 1944, **50**, 344.
11. Moen, J. K., The persistence *in vitro* of the inherent sensitivity to tuberculin of cells from tuberculous animals, *J. Exp. Med.*, 1936, **64**, 943.
12. Favour, C. B., Lytic effect of bacterial products on lymphocytes of tuberculous animals, *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 269.
13. Choucroun, N., Antigenic carbohydrate-lipid isolated from paraffin oil extract of dead tubercle bacilli, *Science*, 1943, **98**, 237.
14. Choucroun, N., Biological effects of a toxic and a sensitizing substance isolated from paraffin oil extract of dead tubercle bacilli, *Science*, 1943, **98**, 327.
15. Choucroun, N., Tubercle bacillus antigens, *Am. Rev. Tuberc.*, 1947, **56**, 203.
16. Choucroun, N., Role joué par le lipopolysaccharide du bacille tuberculeux dans l'hypersensibilité à la tuberculine et dans l'acido-résistance du bacille, *Compt. rend Acad. sc.*, 1948, **226**, 1477.
17. Bloch, H., Studies on the virulence of tubercle bacilli. Isolation and biological properties of a constituent of virulent organisms, *J. Exp. Med.*, 1950, **91**, 197.
18. Bloch, H., Studies on the virulence of tubercle bacilli. The relationship of the physiological state of the organisms to their virulence, *J. Exp. Med.*, 1950, **92**, 507.
19. Spitznagel, J., and Dubos, R. J., A fraction of tubercle bacilli possessing primary toxicity, *J. Exp. Med.*, 1955, **101**, 291.
20. Dubos, R. J., Properties and structures of tubercle bacilli concerned in their pathogenicity, *in* Mechanisms of Microbial Pathogenicity, (J. W. Howie and A. J. O'Hea, editors), Cambridge, England, Society for General Microbiology, 1955, 103.
21. Hanks, J. H., and Evans, B., The action of serum, cells and blood on acid-fast bacteria *in vitro*, *Am. Rev. Tuberc.*, 1940, **41**, 605.
22. Manwaring, W. H., and Bronfenbrenner, J., Intraperitoneal lysis of tubercle bacilli, *J. Exp. Med.*, 1913, **18**, 601.
23. Kirchner, O., Besitzen Tuberkulose- und Nichttuberkulosesera bacterizides Vermogen gegenuber dem Tuberkelbazillus?, *Z. Immunitätsforsch.*, 1932, **74**, 56.
24. Bezançon, F., and Buc, E., La bacteriolyse du bacille de Koch dans les humeurs, *VII Cong. Nat. Tuberc.*, 1931, 108.
25. Baldwin, E. R., Studies on tuberculous serum and the bacteriolysis of bacillus tuberculosis, *J. Med. Research*, 1904, **12**, 215.