THE EFFECT OF TUBERCLE BACILLI ON THE POLYMORPHONUCLEAR LEUCOCYTES OF NORMAL ANIMALS

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Plate 16

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The participation of polymorphonuclear leucocytes in the cellular response of experimental animals to the injection of tubercle bacilli was first convincingly demonstrated in the histological studies of Borrel (1) and has been investigated often during recent years. Thus it has been shown in guinea pigs and rabbits that the bacilli are phagocytized by polymorphonuclear leucocytes immediately upon coming into contact with these phagocytic cells and that the latter undergo a characteristic clumping, apparently as a result of engulfing the bacilli (2, 3).

We have felt that some light might be thrown on the significance of the early cellular response in the pathogenesis of tuberculosis by comparing the effects of virulent tubercle bacilli and of the avirulent variants derived from them on the behavior of polymorphonuclear cells. Any difference in the response of phagocytes to bacilli of different degrees of virulence might conceivably serve as a guide for the analysis of some of the reactions which decide the outcome of the infectious process.

The two cultures H37Rv (virulent) and H37Ra (avirulent) were selected for the first phase of this comparative study because they are variant forms of the same strain of *Mycobacterium tuberculosis hominis* and represent extreme contrasts of virulence and avirulence among true tubercle bacilli. As repeatedly shown, one cell of H37Rv is capable of initiating infection in guinea pigs, whereas H37Ra has never been found to cause progressive disease—however large the dose injected (4). Mice also are susceptible to infection with H37Rv and completely resistant to H37Ra (5) and it was observed by Bloch (6) that, following intraperitoneal injection into these rodents, the avirulent organisms (H37Ra) were much more rapidly removed from the peritoneal cavity than the virulent forms (H37Rv). In an attempt to analyze *in vitro* the mechanism of this differential behavior, Allgöwer and Bloch added virulent bacilli to tissue cultures prepared from the buffy coat of guinea pig blood, and they observed that the migration of the polymorphonuclear cells on the plasma clot was markedly inhibited by the presence of the bacilli (7).

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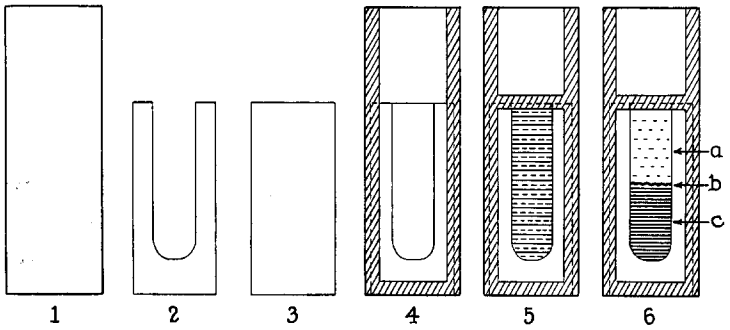
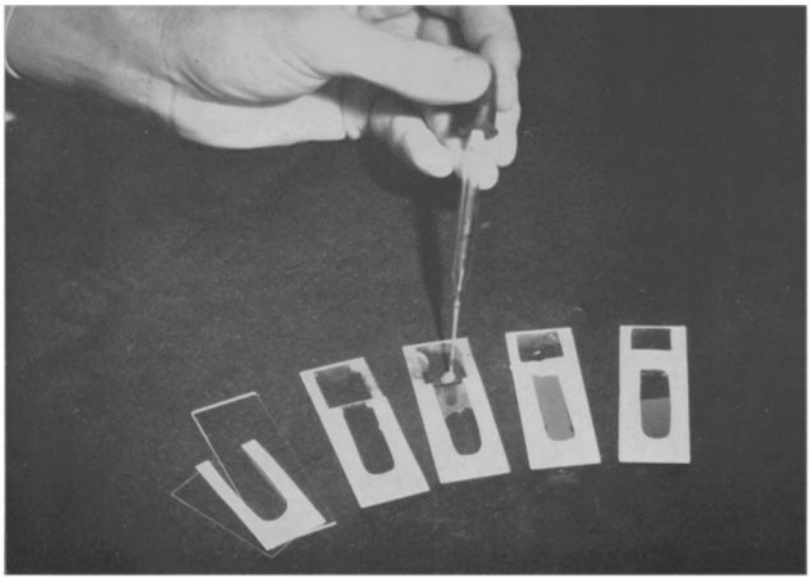


TEXT-FIG. 1. Preparation of slide cell.

1. Microscopic slide (7.5 × 2.5 cm.).
2. Filter paper (Schleicher and Schüll No. 597) cut in U shape.
3. Coverglass No. 1 (22 × 50 mm).
4. Completed cell: Filter paper impregnated with paraffin, sealed between slide and coverglass by heating. Diagonal hatching indicates areas subsequently coated with paraffin.
5. Slide cell filled with blood sample by means of capillary pipette and sealed at top with paraffin.
6. Slide cell after centrifugation. (*a*) Plasma. (*b*) Buffy coat. (*c*) Sedimented red cells.



TEXT-FIG. 2

Steps in the preparation and filling of the slide cell. A cell filled, centrifuged, and ready for incubation is shown at the extreme right.

The experiments to be described in the present paper were designed to investigate the inhibition of migration discovered by Allgöwer and Bloch, as well as other effects of tubercle bacilli on the activities of polymorphonuclear cells.

EXPERIMENTAL

Cultures.—The following cultures[1] of tubercle bacilli were used:

The two extreme variants of the classical H37 human strain derived from a culture of a single cell: H37Rv virulent and H37Ra avirulent. $H37RvL_{12}$ was the 12th transfer in liquid Tween-albumin medium after isolation from the lungs of an infected mouse. $H37RvL_{25}$ was the 25th transfer. $H37Ra_1$ and $H37Ra_2$ are two variant forms isolated from H37Ra and exhibiting slight differences in colonial morphology on oleic acid–albumin agar.

Jamaica, a classical virulent human strain.

R1Rv reported to possess low but definite virulence for normal guinea pigs and to cause fatal disease in silicotic guinea pigs.

Ravenel, a classical virulent bovine strain.

Bovine No. 4, a virulent bovine strain.

A strain of BCG (labelled Phipps in our collection).

A virulent avian strain isolated in our laboratory.

An avirulent avian strain (Kirchberg).

All these cultures were cultivated by weekly transfer in the standard Tween-albumin medium. Their virulence for mice was tested by injecting intravenously 0.2 cc. of 7 to 10 day old diffuse cultures. Survival time, or production of macroscopic lesions and enlargement of spleen within 4 weeks after infection, was used as a measure of virulence.

Preparation of a Slide Cell for the Observation of Migration of Blood Leucocytes.—Allgöwer and Bloch studied the migratory activity of leucocytes in tissue cultures prepared by the classical technique in Carrel flasks (7). This procedure is costly in material and time, requiring in particular large amounts of phagocytic cells. An effort was made therefore to develop a technique that would permit the observation of migration with small amounts of materials, particularly small samples of blood.

Text-figs. 1 and 2 represent the steps in the preparation of a slide cell adapted to the separation from blood of leucocytes, red cells, and plasma. The main walls of the cell consist of a standard microscope slide and a No. 1 coverslip (22 × 50 mm.); narrow lateral walls are provided by a U-shaped piece of filter paper (S & S No. 597) measuring 22 × 50 mm. on the outside and 12 × 40 mm. on the inside. The filter paper saturated with paraffin (m.p. 56–58° C.) is placed between the slide and the coverslip and heated at 60°C. on a hot flat surface. This seals the slide to the coverslip leaving an aperture still open at the top of the U. The free edges of the cooled slide are dipped into paraffin to further seal the coverslip, paper, and slide.

Blood is drawn from the animal to be tested and placed in silicone-coated flasks with an appropriate concentration of heparin which varies with the animal species from which the

[1] The cultures of the H37Rv, H37Ra, R1Rv, and Ravenel strains were received from Dr. W. Steenken of the Trudeau Laboratory, Saranac, New York. The cultures of the Jamaica, Phipps (BCG), bovine No. 4 and Kirchberg strains were received respectively from Dr. J. Freund of the Public Health Research Institute of the City of New York, Dr. J. Aronson of the Phipps Institute, and Dr. W. Feldman of the Mayo Foundation. The culture of avian tubercle bacillus was recovered from the diseased spleen of a tuberculous fowl kindly sent us by Dr. P. J. Brandly of the United States Department of Agriculture. The authors wish to express their gratitude for the help received in this matter.

blood is drawn (Table I). The blood is then transferred to ordinary glass tubes, the material to be tested is added in amounts corresponding to one-tenth the volume of blood, and the mixture is allowed to remain at room or incubator temperature for 15 minutes. A drop of the mixture is then placed at the opening of the slide cell and is allowed to fill it by capillary attraction; the cell is then sealed with paraffin (m.p. 38–40°C.).

The sealed slide cell is placed in a centrifuge cup containing physiological saline up to the level of the blood and is centrifuged at 800 to 1000 R.P.M. for 4 minutes. This operation separates the blood in the chamber into leucocytes, plasma, and red cells. The filling and centrifugation of the slide cell is carried out in a cold room at 0–2°C. to prevent the leucocytes from sticking to the walls of the slide cell.

The slide cell is incubated at an angle of 20° in an incubator at 37°C. With the proper concentration of heparin (Table I) the plasma quickly clots under these conditions. The leucocytes in the buffy coat can be observed under the microscope and their migration followed, after which the preparation can be stained with suitable dyes. This can be done at any time after the plasma has coagulated by removing the coverslip, while the slide is immersed in absolute methyl alcohol. The plasma clot thus remains fixed to the slide and is ready for

TABLE I

The Effect of Various Concentrations of Heparin on the Migration in the Slide Cell of Leucocytes of Chicken, Guinea Pig, Rabbit, and Man*

Origin of leucocytes	Concentration giving complete inhibition	Concentration giving no inhibition	Concentration used in experiments
	mg./cc.	*mg./cc.*	*mg./cc.*
Chicken	0.2	0.12	0.02
Guinea pig	0.2	0.12	0.05
Rabbit	0.1	0.08	0.02
Man	0.1	0.08	0.02

* The heparin (95 units per mg.) was obtained from Connaught Medical Research Laboratories.

staining. Vital staining can be carried out by adding the dye with a micro pipette through the paraffin-sealed end of the slide cell.

Effect of Heparin on the Migration of Leucocytes.—As most of the techniques used in the present investigation for the study of the interrelationships between leucocytes and tubercle bacilli involved the use of heparin, it was important to determine the effect of this substance on the activity of the phagocytic cells.

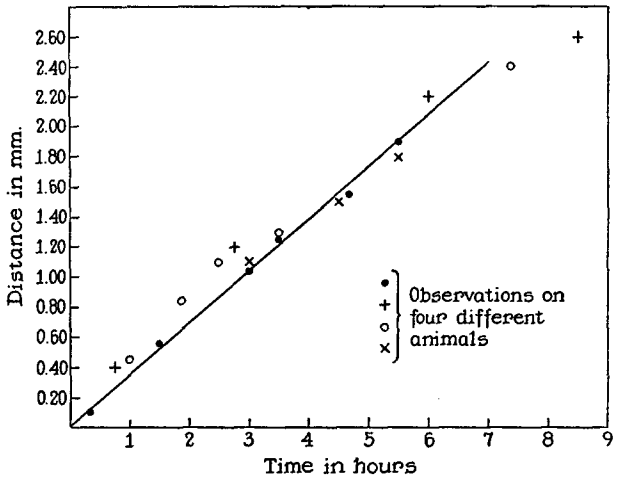
It is known that the phagocytosis of streptococci can be inhibited by concentrations of heparin which vary greatly from one animal species to another (8). Similarly we have observed that the disturbing effect of heparin on the migration of polymorphonuclear leucocytes on plasma clots is also dependent on the origin of the cells.

The results presented in Table I indicate for several animal species the minimal concentration of heparin at which migration was completely inhibited in

the slide cell described above, and the maximal concentration which could be used without disturbing effect. As seen in Table I, the concentrations of heparin used in subsequent experiments were always below the inhibitory dose.

Migration of Leucocytes on Plasma Clots.—In order to determine the reliability of the tests designed to measure the inhibitory effect of tubercle bacilli on the migration of leucocytes, the extent and rate of migration of leucocytes from different animal species were measured in the absence of inhibitory agents.

In Text-fig. 3 is plotted against time the distance of migration of leucocytes obtained from four guinea pigs of the Rockefeller Institute strain. It will be noticed that the rate of migration was remarkably uniform for the four animals and very constant during the first 6 hours of the test. After 12 hours, the rate of



TEXT-FIG. 3. Rate of migration of guinea pig leucocytes on plasma clot (observation made on four guinea pigs of the Rockefeller Institute strain).

forward motion had become distinctly slower. Even at that time, however, the leucocytes still appeared normal according to other criteria, such as random movement, Brownian motion of the intracellular granules, and failure to stain with trypan blue.

Measurements of the greatest distance of migration reached in a 12 hour period revealed variations of 4 to 5 mm. for chicken blood and 3 to 4 mm. for guinea pig (Rockefeller Institute strain) blood. The few samples of rabbit blood and human blood that were tested gave less satisfactory migration.

Effect of Human Tubercle Bacilli on the Migration of Guinea Pig Leucocytes.—The comparative activity of two forms of tubercle bacilli, H37Rv (virulent) and H37Ra (avirulent), in inhibiting the migration of guinea pig blood leucocytes was determined by adding different amounts of these cultures to the slide cells described above. The results of one of these experiments are illustrated

in Fig. 1. It is seen that no inhibition of migration occurred when the culture of the avirulent form (H37Ra) was diluted beyond 1:10. On the other hand inhibition of migration could be detected with dilutions of H37Rv as high as 1:80. Higher dilutions (1:320) of this virulent culture caused a slight stimulation of migration.

A marked clumping of the leucocytes occurred in the slide cells which had received the virulent organisms. Microscopic examination revealed that, in this preparation, most of the bacilli were present in the buffy coat and had been phagocytized. The leucocytes which had engulfed them appeared to adhere to the leucocytes free of bacilli thus bringing about the clumping.

The mere presence of the virulent bacilli in the slide cell was not sufficient to cause inhibition of leucocytic migration. If the bacilli were added to the preparation after centrifugation, when clotting had already taken place, the rate and extent of leucocytic migration were the same as when no bacilli were

TABLE II

Effect of Avian and Human Tubercle Bacilli on the Migration of Guinea Pig and Chicken Leucocytes

Origin of leucocytes	Culture* dilution inhibiting migration			
	H37Rv	H37Ra	Virulent avian	Avirulent avian
Guinea pig	1:160	1:10	—	—
Chicken	1:10	—‡	1:160	—

* Cultures adjusted to equal optical density.

‡ No inhibition with undiluted culture.

present. Similarly, no effect on migration was observed when the bacilli were added to blood which had been held at 0°C. in the slide cell until after centrifugation; these conditions interfered with phagocytosis and allowed most of the bacilli to remain free in the plasma clot.

Effect of Mycobacteria on Leucocytes of Different Animal Species.—As shown in Table II, the migration of chicken leucocytes was inhibited by high dilutions of a culture of avian bacilli recently isolated from the viscera of an infected chicken, but it was not affected by an avirulent avian culture and only a little by a culture of the virulent human strain H37Rv. The migration of guinea pig leucocytes, on the other hand, remained normal in the presence of avian bacilli, virulent or avirulent, but was inhibited by the human culture. The results of this experiment are compatible with the view that the specificity of inhibition of migration reflects the specific pathogenicity of the bacterial agent for the animal species from which the leucocytes are derived. Unfortunately, the failure to obtain reproducible measurements of migration of the blood leucocytes of animals other than guinea pigs and chickens has prevented so far an

adequate study of the relation between pathogenicity and inhibition of migration.

The data summarized in Table III suggest the existence of a correlation between the virulence for albino mice of various strains of mammalian tubercle bacilli and their ability to inhibit the migration of guinea pig leucocytes. It must be emphasized, however, that the number of strains used was far too small to establish the validity of the correlation, and that the strain R1Rv which produces only limited lesions in mice and fails to cause their death proved extremely effective in inhibiting migration.

In the experiments described thus far bacilli and blood leucocytes were brought into contact *in vitro*. As mentioned in the introduction, experiments

TABLE III

Relation of Inhibition of Migration of Leucocytes to Degree of Virulence of Tubercle Bacilli

Bacterial culture	No. of viable units per cc. of culture*	Virulence for mice			Minimal culture dilution inhibiting migration
		Deaths	Survival time	Lesions in survivors	
			days		
Human virulent $H37RvL_{12}$	28×10^5	10/10	20 ± 3.2		1/200
" " $H37RvL_{25}$	20×10^5	4/10		++++	1/200
" " Jamaica	28×10^5	10/10	29 ± 10		1/200
Bovine " Ravenel	40×10^5	10/10	20 ± 9.1		1/200
" " No. 4	9×10^5	10/10	20 ± 2.7		1/100
Human attenuated R1Rv	56×10^5	0/10		++	1/50
Bovine " BCG (Phipps)	18×10^5	0/10		+	1/10
Human avirulent $H37Ra_1$	18×10^5	0/10		0	—‡
" " $H37Ra_2$	30×10^5	0/10		0	—‡

* Determined by plating on oleic acid–albumin agar.

‡ No inhibition with undiluted culture.

by Bloch (6) had suggested that inhibition of migration of polymorphonuclear leucocytes occurred when virulent tubercle bacilli were introduced into the peritoneal cavity of the mouse. The following experiment reveals that injection of virulent tubercle bacilli into living guinea pigs affects the migratory activity of leucocytes of the bone marrow removed from these animals.

Five cc. of a 7 day old culture of tubercle bacilli (H37Rv or H37Ra) in Tween-albumin medium (containing 5×10^7 viable units per cc.) was injected into the left ventricle of the heart of adult guinea pigs. Five minutes, and again 3 hours later the sternal and femoral bone marrow (known to contain large numbers of polymorphonuclear leucocytes) was removed and placed on a surface prepared by dissolving 0.2 gm. of bovine fibrinogen in 10 cc. of 30 per cent guinea pig serum diluted in Tyrode solution.

The polymorphonuclear leucocytes of the marrow of untreated animals exhibited active migration, forming in 18 hours at 37°C. a 3 to 5 mm. halo of

cells about the explant. The leucocytes of the animal injected with H37Ra also migrated at an active rate. There was however marked inhibition of migration in the explants from animals which had received the virulent H37Rv, notably when the bone marrow had been collected 3 hours after injection. Interestingly enough, this impairment of migration occurred despite the fact that only few microorganisms could be seen in stained preparations of the bone marrow (one organism per 8 to 10 microscopic immersion fields).

Chemotactic Effect of Virulent Tubercle Bacilli.—It has long been known that some of the constituents of tubercle bacilli—particularly the tuberculo-phosphatide fraction—exert a positive chemotactic effect on polymorphonuclear cells (9). An experiment was therefore instituted to test whether the differences in ability to inhibit leucocytic migration could be traced to differences in the chemotactic powers of the virulent and avirulent variants.

Sternal and femoral bone marrow of normal guinea pigs was used as a source of leucocytes. The chemotactic influence was determined by exposing the bone marrow cells to virulent and avirulent bacilli in agar blocks or in capillary tubes according to the technique described by Chambers and Grand (10).

Under the conditions of this experiment, no difference could be detected in the pattern of migration of the leucocytes toward H37Rv or H37Ra, either in the capillary tubes or in the agar blocks—a fact suggesting that the chemotactic attraction was of the same order for the two bacterial forms and could not account for the differences in migratory activity observed in the experiments described earlier in this report.

Failure to Demonstrate a Toxic Effect of Tubercle Bacilli on Polymorphonuclear Cells.—In the absence of a selective chemotactic effect of virulent tubercle bacilli, the inhibition of migration appears most readily explained in terms of a toxic influence of the bacilli on the phagocytes. Experiments were therefore instituted to search for more objective evidence of this hypothetical toxic effect.

A peritoneal exudate rich in polymorphonuclear leucocytes was obtained from guinea pigs by the injection of sodium caseinate. The caseinate was prepared by dissolving (at pH 7.0) 72 gm. of casein (No. 453, Casein Co. of America) in 1000 cc. physiological saline by addition of 2 N NaOH. The solution was then autoclaved and stored. Ten cc. of this solution was injected intraperitoneally into adult guinea pigs and the resulting exudate collected aseptically 12 to 14 hours later in the presence of heparin in a final concentration of 1:5000.

Samples of this leucocyte preparation resuspended in 20 per cent guinea pig serum diluted in Tyrode solution, were placed in silicone-coated glass tubes to some of which were added various amounts of cultures of tubercle bacilli diluted in Tween-albumin medium. The mixtures were slowly rotated at 37°C. by fastening the tubes to the periphery of a 24 cm. wheel which was turned at 4 R.P.M. by an electric motor (11). Total white counts were taken at intervals of 3 and 8 hours. After each interval of time the cells were stained with methylene blue (12) and trypan blue (13).

The results are summarized in Table IV.

As seen in Table IV the determination of the total number of leucocytes or of the percentage of leucocytes which became stainable with trypan blue, after various periods of incubation, failed to reveal any evidence of toxic effect of the bacilli on these cells.

Attempts were also made to determine the effect of engulfment of virulent and avirulent tubercle bacilli on the metabolism of guinea pig leucocytes (from peritoneal exudates). Observation of reduction of methylene blue in Thunberg tubes, and of oxygen uptake in a Warburg respirometer, failed to reveal any depressing effect of the bacteria on the metabolism of the leucocytes.[2]

In the absence of direct evidence of an effect on the viability or metabolism of leucocytes, an effort was made to determine whether tubercle bacilli did exert

TABLE IV

Effect of Phagocytosis of Virulent (H37Rv) and Avirulent (H37Ra) Tubercle Bacilli on the Viability of Polymorphonuclear Cells

Phagocytes resuspended in	Time of Incubation					
	0		3 hrs.		8 hrs.	
	Total count	Stainable with trypan blue	Total count	Stainable with trypan blue	Total count	Stainable with trypan blue
		per cent		*per cent*		*per cent*
30 per cent serum in Tyrode solution	28,500	0.1	28,200	0.1	28,750	0.4
Tween-albumin medium	29,450	0.2	29,600	0.2	25,500	0.6
Undiluted H37Rv culture	30,700	0.1	33,550	0.1	28,000	0.5
H37Rv culture diluted 1:10	29,350	0.1	28,200	0.1	20,500	1.2
" " " 1:100	29,500	0.2	29,000	0.0	23,000	0.8
Undiluted H37Ra culture	29,500	0.1	32,000	0.1	24,200	0.8
H37Ra culture diluted 1:10	28,200	0.0	26,900	0.0	22,700	0.8
" " " 1:100	26,000	0.1	25,450	0.2	22,200	0.4

some influence on the biological activity of these cells. The ability of leucocytes to phagocytize and kill rough pneumococci was selected as a criterion of physiological function.

Suspensions of leucocytes (from guinea pig peritoneal exudates resuspended in 20 per cent guinea pig serum diluted in Tyrode solution) were placed in silicone-coated glass tubes with various dilutions of cultures of H37Rv and H37Ra in Tween-albumin medium. These mixtures were slowly rotated as described above. After 2 hours and 4 hours a suspension of rough pneumococci (strain D39R) was added. The rotation was continued and the number of surviving pneumococci determined after 6 hours by plating on blood agar. Stains were made to determine the distribution of bacilli in the leucocytes.

In this case again, the results failed to give any evidence that the presence of tubercle bacilli interfered in any way with the ability of leucocytes to kill the

[2] These tests were carried out by Dr. W. Emanuel Suter.

pneumococci added to the system. Moreover it was seen in stained preparations that leucocytes loaded with tubercle bacilli had been capable of phagocytizing large numbers of pneumococci, and therefore had retained much of their physiological activity.

DISCUSSION

The experiments reported in the present paper confirm Allgöwer and Bloch's finding that polymorphonuclear cells which have engulfed virulent tubercle bacilli lose part or all their ability to migrate on plasma clots. Surprisingly enough this inhibitory effect of the bacilli was not accompanied by any other evidence of toxicity that we have been able to recognize through tests directed to that point.

Microscopic examination failed to give any indication that the viability of polymorphonuclear leucocytes was affected by prior engulfment of tubercle bacilli. Moreover, physiological activity remained unaltered whether it was measured by uptake of oxygen or by ability to phagocytize and destroy pneumococci. Other than change in the rate of migration, the only alteration which was observed in leucocytes that had engulfed virulent tubercle bacilli was their increased tendency to adhere to other leucocytes, as if their surface had become sticky. Indeed, one could assume that the depression of migratory activity was due to such a change in the surface properties of the leucocytes.

Whatever the mechanism of the effect by which tubercle bacilli interfere with the migration of polymorphonuclear cells, this effect appears to bear a certain, although ill defined, relation to pathogenicity. Thus, in a general way, migration of guinea pig leucocytes could be inhibited with amounts of virulent mammalian bacilli smaller than the amounts required of the avirulent bacterial variants. On the other hand virulent mammalian bacilli were ineffective in inhibiting the migration of the leucocytes of chickens (species for which they are not pathogenic) whereas virulent avian bacilli inhibited avian cells but not guinea pig cells (Table II). Although the correlation between pathogenicity and inhibition of migration thus appears suggestive, demonstration of its validity would require a quantitative investigation of the effects of many bacterial strains of known pathogenicity on the leucocytes of many various animal species.

It is well known that the phagocytosis of tubercle bacilli by polymorphonuclear leucocytes represents only one of the first phases of the tuberculous infection and that the mononuclear leucocytes soon come to play a dominant role in the pathogenesis of the disease. Although the present study was not concerned with the effects of tubercle bacilli on mononuclear cells it appears worth pointing out that, in tissue cultures at least, bacilli and macrophages are able to coexist in almost perfect symbiosis. Bacillary multiplication can occur to an enormous extent and the bacilli accumulate in large numbers both extracellu-

larly and intracellularly, without causing any detectable damage to the mononuclear phagocytes or to the fibroblasts in the tissue cultures (14). Thus it appears that, whatever the nature of the mechanism by which virulent tubercle bacilli are able to cause disease in normal susceptible hosts, bacillary invasion does not depend upon a gross cytotoxic effect on the non-sensitized phagocytic cells.

SUMMARY

A description is given of a slide cell whereby the rate of migration of very small amounts of leucocytes can be followed and measured.

The migration of polymorphonuclear leucocytes was found to be inhibited by virulent tubercle bacilli pathogenic for the class of animal (mammal or bird) from which the leucocytes were obtained; it was not affected by the avirulent variants of these microorganisms, or by bacilli pathogenic for animals of the other class.

Tests failed to disclose that the inhibition of leucocytic migration resulted from any gross damage caused by the bacilli to the leucocytes.

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EXPLANATION OF PLATE 16

FIG. 1. Effect of H37Rv (virulent) and H37Ra (avirulent) on migration of leucocytes. Clumping and inhibition of migration of leucocytes are apparent in the slide cell containing H37Rv.

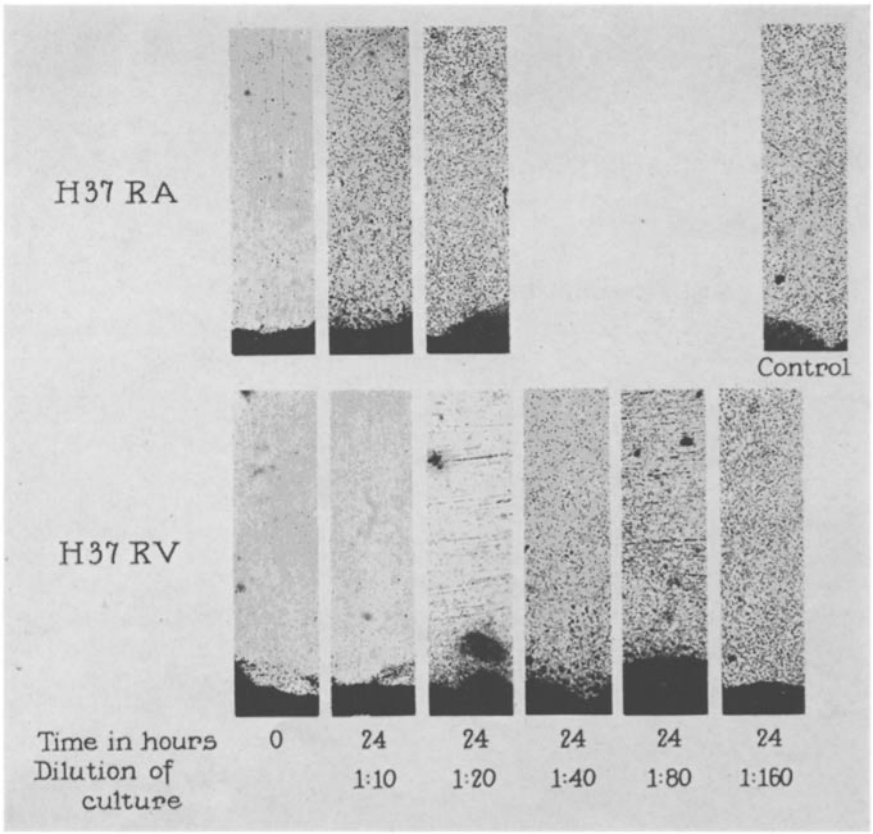

FIG. 1

(Martin *et al.*: Tubercle bacilli and leucocytic cells)