ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

II. Correlation between Changes in Bacterial Surface Properties and in Phagocytosis Caused by Normal and Immune Sera.

By BALDUIN LUCKÉ, M.D., MORTON McCUTCHEON, M.D., MAX STRUMIA, M.D., and STUART MUDD, M.D.

(From the Department of Pathology, and the Henry Phipps Institute, University of Pennsylvania, Philadelphia.)

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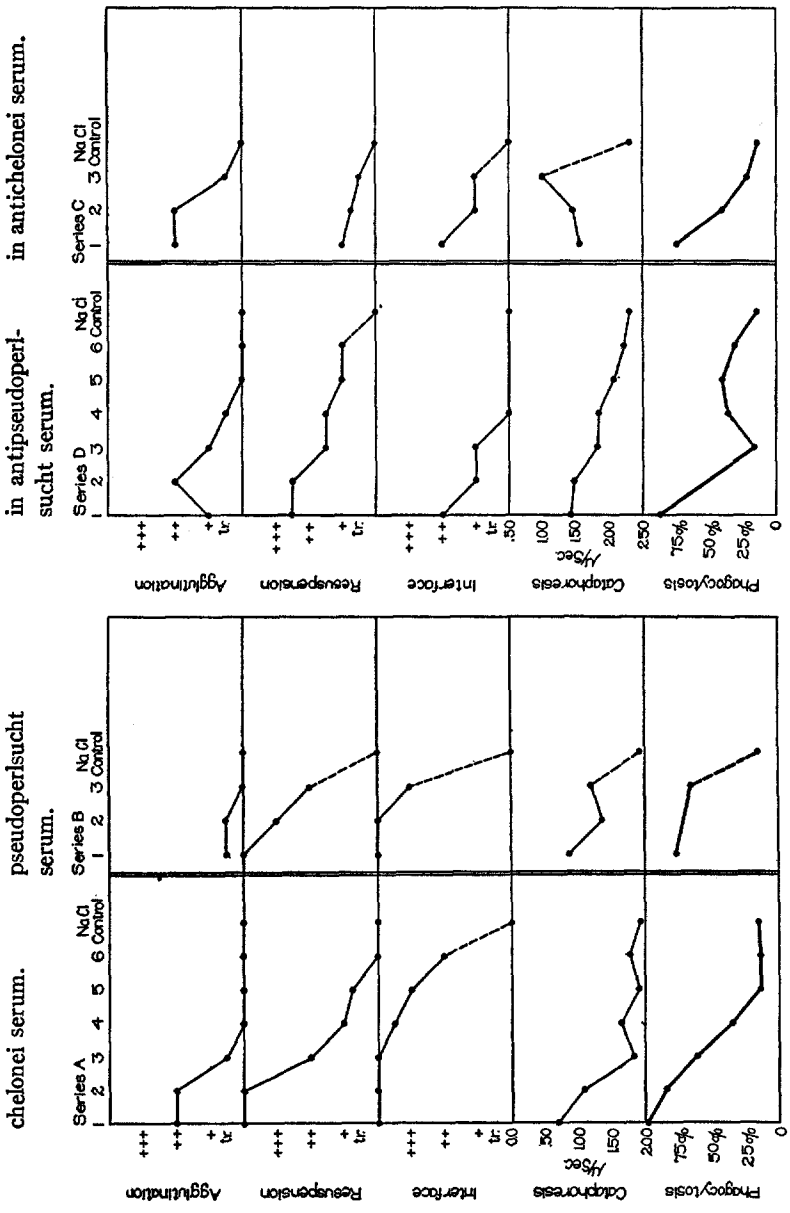
The sera of rabbits under immunization have been shown in the preceding paper (1) to cause parallel effects on the surface properties and the phagocytosis of acid-fast bacteria. Is this correspondence between the serum effects on surface properties and on phagocytosis a constant and necessary relation, or do circumstances exist in which the two effects are dissociated? It can be said at once that, in our experiments at least, whenever a serum has promoted phagocytosis it has invariably also altered the properties of the bacterial surfaces; and the surface and phagocytic effects have been in rough quantitative correspondence at least. Exceptionally circumstances have been found in which sera have altered bacterial surface properties, but have not increased phagocytosis. These failures of correspondence have all been with heated or aged sera or with sera of another species than the species from which the leukocytes were obtained.

Bacteriotropins.

Freshly drawn immune sera have regularly shown parallel bacteriotropic and bacterial-surface effects. An illustrative experiment is given in Text-fig. 1. The intensities of the several reactions are plotted as ordinates, and the final serum dilutions as abscissæ. It is seen that each serum when acting on its homologous strain caused increased cohesiveness (as indicated by the positive resuspension and interface



TEXT-FIG. 1. Homologous and cross reactions between turtle and pseudoperlsucht bacilli and their corresponding rabbit immune sera. Fresh sera heated to 56°C. for 30 minutes. Broken lines indicate that the titer was not reached. Numbers on horizontal axis indicate serum dilutions in powers of 4; thus 3 indicates a dilution of $1:4^3$ or 1:64. There is a general correspondence between bacterial surface changes and phagocytosis.

reactions), altered wetting properties (interface reaction), decreased surface potential difference (cataphoresis), and increased phagocytosis. The antipseudoperlsucht serum caused strong surface and bacteriotropic effects when used with the turtle bacillus (*M. chelonei*), and antichelonei serum gave moderate surface and bacteriotropic effects with the pseudoperlsucht bacillus.

The familiar fact that bacteriotropic action is exerted upon the bacterium not upon the leucocyte is demonstrated in Table I. In this experiment the bacteria were sensitized and then washed and suspended in saline solution. It is seen that phagocytosis is efficiently promoted by sensitization even in the absence of the sensitizing serum. The strains used were an avian and a bovine tubercle bacillus. Cross reactions are here almost as strong as those between homologous antigen and antibody; and again bacterial surface changes and increase in phagocytosis are in close correspondence.[1]

Aged Immune Sera.—Rabbit immune sera which had been kept in the ice-box without preservative for about 16 to 18 months were kindly put at our disposal by Dr. J. D. Aronson. These had been kept in large test tubes stoppered with cotton; accordingly they had undergone some evaporation. Moreover a heavy greyish precipitate had formed in the bottom of each, and most of the sera showed some opalescence which could not be cleared by centrifugation. Typical effects produced by such sera are shown in Text-figs. 2 and 3.

Prague bacillus treated with homologous antiserum exhibits characteristic surface changes (Series A), (Text-fig. 2). Phagocytosis is also increased but only in high dilutions of serum, reaching its maximum at approximately 1:1000 and 1:4000 dilution. This surprising phagocytic prezone cannot be explained satisfactorily by postulating injury of the leucocytes by the aged serum, as is shown by Series C, in which the same anti-Prague serum caused a moderate increase in phagocytosis without prezone, with milk bacillus, in correspondence with moderate bacterial surface changes.[2] The anti-milk-bacillus serum with homologous antigen caused strong bacterial surface

[1] A graph of this experiment has appeared in Colloid symposium monographs (2).

[2] The tubes of Series C were contaminated with a coccus. This may have been responsible for the prezone in the resuspension reaction, which is not a usual finding.

TABLE I.

Homologous and Cross Reactions between M. avium (Prague Strain) and M. tuberculosis (Bovine III Strain) and Their Corresponding Rabbit Immune Sera. Serum Dilutions Are Given as Powers of 4.

In this experiment sensitized bacteria were washed before phagocytosis. It is seen that even in the absence of free serum there was a high degree of phagocytosis.

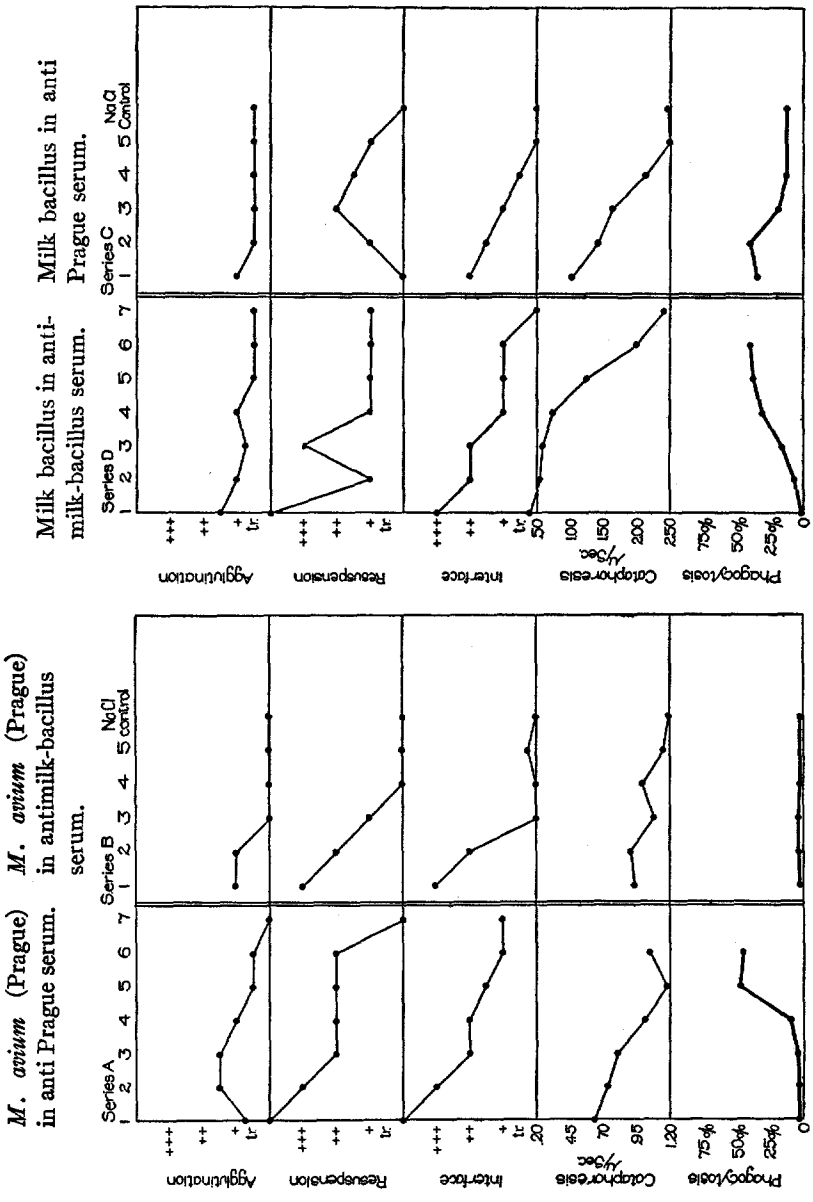
	Serum dilutions								Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	1:65,536	
Series A. Prague in Anti-Prague Serum.									
Agglutination	++++ to +++	++++ to +++	++++ to +++	+	sl. tr.	sl. tr.	sl. tr.	sl. tr.	sl. tr.
Resuspension	++++	++++	+++	++	0	tr.	0	0	0
Interface	++++	++++	++ to +	+	tr.	0	—	—	0
Cataphoresis, μ/*sec*	0.34	0.21	0.45	0.70	0.83	0.86	0.93	—	1.02
Phagocytosis, *per cent*	92	97	99	94	52	7	7	6	3
Series B. Prague in Anti-Bovine III Serum.									
Agglutination	++++ to +++	++++ to +++	+++	+	+ to tr.	tr.	—	—	sl. tr.
Resuspension	+++ to ++	++++ to +++	+++ to ++	++ to +	0	0	—	—	0
Interface	+++	+	tr.	0	—	—	—	—	0
Cataphoresis, μ/*sec*	0.24	0.39	0.65	0.79	0.89	0.91	—	—	0.89, 0.98
Phagocytosis, *per cent*	73	64	79	72	44	20	—	—	4

Series C. Bovine III in Anti-Prague Serum.

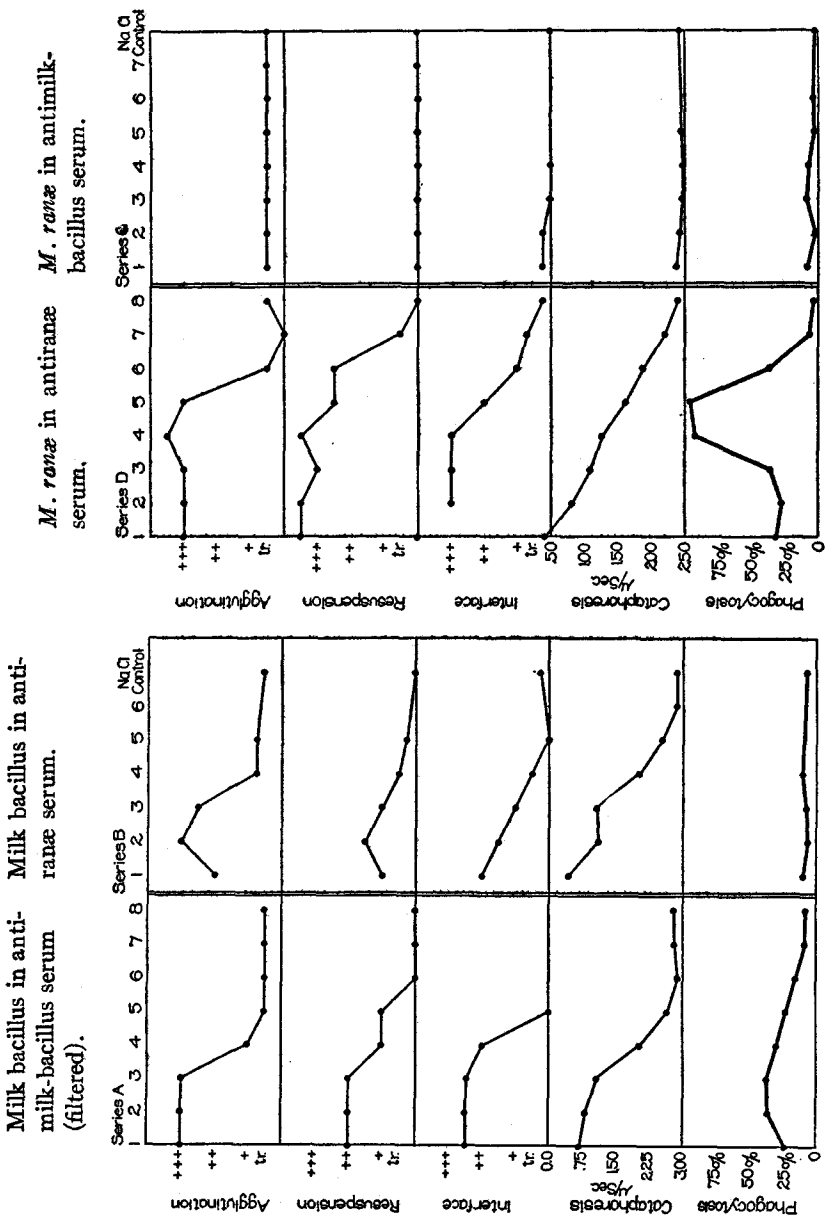
Agglutination.....	+++ to ++	+++ to ++	++	+	sl. tr.	sl. tr.	—	—	sl. tr.
Resuspension.....	++++	+++	++	+	0	0	—	—	0
Interface.....	+++	+++	++	+	tr.	0	—	—	0
Cataphoresis, μ/*sec*.....	0.35	0.47	0.92	1.40	1.33	1.53	—	—	1.52
Phagocytosis, *per cent*.....	44	46	28	43	35	9	—	—	3

Series D. Bovine III in Anti-Bovine III Serum.

Agglutination.....	++++ to +++	++++ to +++	+++	++	sl. tr.	sl. tr.	sl. tr.	sl. tr.	sl. tr.
Resuspension.....	++++	++++	+++	++	0	0	0	0	0
Interface.....	++++	+++	+++ to ++	++ to +	+	tr.	0	—	0
Cataphoresis, μ/*sec*.....	0.08	0.17	0.31	1.18	1.17	1.53	1.46	1.50	1.69
Phagocytosis, *per cent*.....	47	44	45	20	31	15	7	4	6



TEXT-FIG. 2. Homologous and cross reactions between an avian tubercle bacillus and a saprophyte and their corresponding rabbit immune sera. Sera aged about 16 months. Symbols as in Fig. 1. Note phagocytic prezones.



TEXT-FIG. 3. Homologous and cross reactions between frog and milk bacilli and their corresponding rabbit immune sera. Sera aged about 16 months, and filtered before use. Symbols as in Fig. 1. Note phagocytic prezones.

changes and enhanced phagocytosis, but again with a striking phagocytic prezone. This serum, on the other hand, failed to increase phagocytosis of Prague bacillus, although it caused moderate surface changes (Series B).

Anti-milk bacillus serum from the same source as in Text-fig. 2 was passed through a Seitz asbestos laboratory filter disc and used in the experiment represented by Text-fig. 3. It is seen that the percentage phagocytosis caused by the filtered serum is somewhat less than that caused by the unfiltered, and the phagocytic prezone has been almost abolished by the filtration (Series A). The milk bacillus serum was practically without effect either on the surface properties or the phagocytosis of frog bacillus (*M. ranæ*, Series C). Anti-frog-bacillus serum, which also had been filtered through a Seitz disc, caused surface changes and increased phagocytosis, with prezone, with its homologous antigen (Series D). The anti-frog bacillus serum caused moderate surface reactions but no increased phagocytosis with the heterologous organism (Series B).

Two conditions have been found, then, in which correspondence may fail between the effects produced by aged sera on the bacterial surface properties and on phagocytosis. Save for them the correspondence of surface and phagocytic effects has been satisfactory even with aged immune sera. These conditions are:

1. The aged sera in all of 13 homologous series gave phagocytic prezones. Maximum phagocytosis was reached in serum dilutions which might be as high as 1:1000; phagocytosis in the lesser serum dilutions was far below the maximum or even absent. Prezones comparable to this were not found with the surface reactions.

2. The aged sera sometimes produced surface changes but no phagocytosis when reacting with heterologous bacterial strains. Four out of 11 series gave this result; in the other 7 heterologous series correspondence between bacterial surface changes and phagocytosis was satisfactory.

The Phagocytic Prezone with Aged Immune Sera.—Phagocytic prezones occurred, as has been said, in all of the homologous series with the aged immune sera; only one prezone occurred in a heterologous series. Moreover we have two clear experiments in which an aged serum gave a striking prezone with its homologous bacterial strain,

but caused phagocytosis without prezone with a heterologous strain. The first example is given by Series A and C of Text-fig. 2. The second is given by Series A and C of Table II. Series B and D of Table II seem to be a third example, but this is less clear because of the irregularity of the phagocytosis in Series D.

The results just cited indicate that the phagocytic prezone cannot be chiefly due to injury of the leucocytes by the aged sera or to other non-specific effect, but must be an expression of the properties of the specific combination of bacteria and sensitizing substances. The evidence leading to this conclusion is strengthened by the observation that a phagocytic prezone may persist after washing the sensitized bacteria. In 4 series in which the sensitized bacteria were washed, some prezone persisted in 3; in the fourth series phagocytosis was practically abolished by washing. Two of the persistent prezones were, however, considerably diminished by the washing; one of these is shown in Table III.

It is important to bear in mind that these phagocytic prezones with aged sera, however interesting and perplexing from a theoretical standpoint, are a distinctly artificial phenomenon. Such prezones have practically not occurred, at least in our experience, with sera used within a few days of drawing the blood, whether the sera have been heated to 56°C. for 30 minutes or have been used unheated. Such fresh sera have almost invariably shown phagocytosis at or near maximum in the highest serum concentration used in this work, namely a final concentration of 25 per cent serum.

The most marked prezone found with freshly prepared sera occurred in Rabbit 20-02, at the end of its 3rd week of immunization with Arloing bacillus (1); the percentages of phagocytosis in the successive tubes in this experiment (the dilution factor was 0.25 as usual) were 67, 72, 89, 97, 81, 26, 10, 11, and control 11 per cent. This prezone was not present with the serum of the same rabbit the week before or after this experiment. Moreover the leucocytes were noted as agglutinated in the first tubes of this series; this probably explains the apparent prezone.

There are good grounds for supposing that the protective power of some immune sera depends largely upon their efficiency in promoting phagocytosis (3). The abnormal bacteriotropic behavior of the aged sera used in our experiments suggests a danger in dispensing old antibacterial sera for clinical use.

TABLE II.

Homologous and Cross Reactions between M. avium (Arloing Strain) and M. tuberculosis (Bovine III Strain) and Their Corresponding Rabbit Immune Sera.

Prezones in phagocytosis are shown in homologous reactions but not in cross reactions.

	Serum dilutions							Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	
Series A. Arloing in Anti-Arloing Serum.								
Agglutination	+++	+	+++	+++	+	0		0
Resuspension	+++ to ++	+++	++++	+++ to ++	+ to tr.	tr.		0
Interface	++	++++	+++ to ++++	++	+	tr.		0
Cataphoresis, μ/*sec.*	1.27	1.73	1.80	1.96	1.65	1.91		1.64
Phagocytosis, *per cent.*	22	22	72	96	81	40	11	8
Series B. Arloing in Anti-Bovine III Serum.								
Agglutination	+++	+++	+++	++	0	0		0
Resuspension	++++ to +++	++++ to +++	+++	tr.	0	0		0
Interface	+++	++	++ to +	+	+ to tr.	0	0	0
Cataphoresis, μ/*sec.*	0.95	1.20	1.47	1.81	1.80			1.77
Phagocytosis, *per cent.*	53	26	25	9	11	10		—

Series C. Bovine III in Anti-Arloing Serum.

Agglutination.....	+	+	tr.	0	0	0		0
Resuspension.....	++	+	++	+ to tr.	0	0		0
Interface.....	+++	++ to +++	++	++	tr.	0		0
Cataphoresis, μ/*sec*.....	1.54	1.16	1.15	1.52	1.26			1.54
Phagocytosis, *per cent*..	24	19	10	12	13	9		1

Series D. Bovine III in Anti-Bovine III Serum.

Agglutination.....	+	+	+	+	tr.	0		0
Resuspension.....	+	++	++	++	++ to +	tr.		0
Interface.....	++	+++	+++	+++	+	tr.		0
Cataphoresis, μ/*sec*....	0.97	0.94	1.11	1.69	1.14	1.25	1.33	1.64
Phagocytosis, *per cent*..	15	11	14	11.5	29	3	2	—

TABLE III.

The Effect on Surface Properties and on Phagocytosis of Washing Bacteria Sensitized with Aged Serum. M. avium (Arloing Strain) Was Sensitized with Homologous Aged Rabbit Serum. In Series A Determinations Were Made in the Presence of Serum Dilutions; in Series B Sensitized Bacteria Were Washed to Remove Free Serum.

It is seen that the intensity of the phagocytic prezone is diminished by washing the bacteria.

	Serum dilutions							Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	
Series A (in Serum Dilutions)								
Agglutination	+++ to ++	+++ to ++	+++	++++ to +++	tr. to 0	0	0	0
Resuspension	++	++	+++	++++	+	tr.	0	0
Interface	+++	+++	++++	++	+	+		0
Cataphoresis, μ/*sec.*	0.93	1.47	1.69	1.76	1.50	1.42	1.45	1.61
Phagocytosis, *per cent.*	25	28	69	90	78	45	7	8
Series B (Sensitized, Washed)								
Agglutination	—	—	—	—	—	—	—	—
Resuspension	++ to +	++	+++	+++	++ to +	+ to 0	0	0
Interface	+++ to ++	++++	++++ to +++	+++	+ to tr.	±	0	±
Cataphoresis, μ/*sec.*	0.28	0.33	0.55	1.25	1.42	1.57	1.63	1.64
Phagocytosis, *per cent.*	55	54	62	65	85	73	32	—
Series C (Sensitized in 1:256 Serum, Then Made Up in Serum Dilutions)								
Phagocytosis, *per cent.*	30	29	68	87	93	93	85	—

Opsonins.

Rabbit Sera.—With fresh, unheated sera of normal rabbits there has been excellent parallelism between the bacterial surface changes and opsonization. Bacterial cohesion has been increased, surface potential difference reduced, wetting properties altered and the bacteria have been prepared for phagocytosis. The serum concentrations required to give these effects are of course much higher than with immune sera.

The lowest concentrations of normal serum which promoted phagocytosis were for sensitized, washed bacteria: 1:5, 1:5, 1:5, 4:5, 1:5, 4:5, and 1:5; one series in which the bacteria were washed failed to give phagocytosis even after sensitization with 80 per cent serum. For bacteria exposed to leucocytes in the presence of fresh normal sera the titers were somewhat higher, *i.e.*, 1:16, 1:16, 1:64 and 1:16. The resuspension and cataphoresis titers have run on the average a little higher than the phagocytosis titers, the interface titers about the same, although all titers with these concentrated normal sera have tended to be irregular.

With the sera of normal rabbits inactivated by heating to 56°C. for 30 minutes correspondence between bacterial surface changes and phagocytosis has not been good. In 7 series of bacteria sensitized with heated normal sera and washed, phagocytosis was not induced in any, whereas the surface changes were present, although to a less degree than after sensitization with unheated serum. In 28 series in which the bacteria were exposed to leucocytes in the presence of heated normal rabbit serum phagocytosis was induced in only about 16 series, whereas the bacterial surface properties were altered to some degree in practically all.

Human Sera.

Bacteria have been sensitized with fresh human sera, washed with 0.85 per cent sodium chloride solution, and tested for surface changes and for phagocytosis by rabbit leucocytes. The sera tested were from 7 non-tuberculous individuals, from 5 cases of pulmonary and 6 of bone or joint tuberculosis, as indicated in the protocols below. The characteristic changes in bacterial surface properties were regularly produced by these sera. Qualitatively the changes were not distinguishable by the surface reactions from those produced by rabbit sera. Quantitatively the bacterial surface changes varied with the different

sera, but were usually a little greater than those produced by normal unheated rabbit sera. Yet in no case was a clearly demonstrable increase in phagocytosis by rabbit leucocytes caused by sensitization with human serum.

Patients with Pulmonary Tuberculosis.—(Sera made available through kindness of Dr. I. Kaufman.)

A. L., female. Pulmonary tuberculosis, moderately advanced, moderate symptoms, sputum positive; tuberculosis complement fixation, ++++; Wassermann negative. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with *M. tuberculosis* (Bovine III).

E. G., female. Pulmonary tuberculosis, moderately advanced, slight symptoms, sputum positive; tuberculosis complement fixation, negative; Wassermann negative; tuberculin, 0.00001 cc. O. T., ++. Unheated serum tested in dilutions of 1:4, 1:16, 1:64 and 1:256 with Bovine III.

M. C., female. Pulmonary tuberculosis with apical râles; far advanced; severe symptoms; sputum positive. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

B. R., female. Pulmonary tuberculosis with apical râles; far advanced, moderate symptoms. Tuberculosis complement fixation, negative; Wassermann negative. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

G. H., female. Apical tuberculosis; tuberculosis complement fixation ++++; tuberculin, 0.00001 cc. O.T., +. Wassermann ++++. Unheated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

Surgical Tuberculosis.—(Sera made available through kindness of Dr. George Wagoner.)

Sera of A. A. and C. P. tested unheated in dilutions of 1:4, 1:16, 1:64, 1:256 and 1:1024. Other sera tested, unheated, in dilutions of 1:4 and 1:16. All sera tested, heated, in dilutions of 1:4. Antigen for all series, *M. tuberculosis* (Bovine III).

A. A., male. Pott's disease of 4th, 5th, 6th and 7th dorsal vertebræ.

C. P., male. Pott's disease of 5th and 6th dorsal vertebræ.

I. L., male. Lumbo-sacral spondylitis, probably tuberculous, numerous sinuses, pulmonary tuberculosis (?).

J. S. Tuberculous left hip with sinuses; old pulmonary tuberculosis.

A. S. Tuberculous spine and shoulder with sinuses.

H. S. Tuberculous right wrist (?).

Non-Tuberculous Individuals.—

Sera from 6 persons who came into the chest clinic but were shown by physical and laboratory examination to be free from clinical tuberculosis. Serum from a healthy university student. Tests as above with Bovine III or an avian tubercle bacillus as antigen.

These experiments failed to give evidence that any of the reactions used afforded a satisfactory means of distinguishing the sera of tuberculous from those of non-tuberculous individuals.

It is to be emphasized that the bacteria sensitized with human sera were in all cases washed before testing for phagocytosis in order to exclude the possibility of a direct toxic effect of human serum on the rabbit leucocytes. The leucocytes were shown to be capable of phagocytosis by other series in the same experiments in which sensitization was produced by rabbit sera. A further experiment was performed in which bacteria were first sensitized with unheated normal rabbit serum, then washed and resuspended in 1:500 human serum before testing for phagocytosis. This concentration of human serum was considerably higher than the amount which could have remained after washing of the bacteria sensitized even with the highest concentrations of human serum. Phagocytosis of the bacteria sensitized with rabbit serum occurred nevertheless in the presence of the 1:500 human serum.

The rabbit polymorphonuclear leucocytes can then differentiate with precision bacteria sensitized with human serum from those sensitized with rabbit serum, whereas our physical-chemical tests fail to make this differentiation. We believe phagocytosis to be dependent upon the spreading of the leucocyte about the bacterium primarily under the action of surface forces (1). This capacity of a leucocyte to differentiate the sensitizing substances of two mammalian species[3] implies a very striking selectivity in the spreading or wetting requirements of the leucocyte, which, however, is not wholly without analogy. Thus sponges have been separated into their component cells by straining through cloth and the separated cells of different species have been mixed together. The cells creep about by amœboid motion until they meet and fuse with other cells of the same species. Syncytia composed of cells of each species but not of mixed species are

[3] However, we have obtained abundant phagocytosis by rabbit leucocytes of tubercle bacilli sensitized with hyperimmune antituberculous goat sera and washed with saline. Robertson and his collaborators obtained phagocytosis of pneumococci by rabbit leucocytes suspended in rabbit serum to which human serum containing antipneumococcus antibodies had been added (3). Normal serum added to such rabbit serum leucocyte mixtures did not cause phagocytosis (3, 4).

thus formed (5). The spreading of fluids, in general, is known to be to some extent selective, and to depend both on the properties of the spreading surface and the surface spread upon (6).

In the experiments with normal rabbit sera, then, excellent correspondence has been obtained between changes in bacterial surface properties and opsonic effects so long as fresh, unheated sera have been used. With heated normal rabbit serum or with human serum, heated or unheated, surface changes have been produced but phagocytosis has often failed. It would seem then that the requirements for spreading of the leucocyte surface are so delicate that discrimination occurs between sensitizing substances deposited on the one hand by fresh immune rabbit sera and fresh unheated normal rabbit sera and on the other hand by aged immune sera, heated normal rabbit sera and human sera. The fact may once again be emphasized that the failures of correspondence between bacterial surface changes and phagocytosis have all been failures of phagocytosis, and have all been found under extremely artificial conditions. Correspondence between changes in bacterial surface properties and increase in phagocytosis has been excellent when conditions *in vitro* have been made to imitate as nearly as possible conditions *in vivo*.

CONCLUSIONS.

The work reported in this and in previous papers (1, 7) demonstrates the following relations for acid-fast bacteria and rabbit polymorphonuclear leucocytes:

1. The combination of a substance or substances present in fresh immune rabbit serum, heated or unheated, or in fresh unheated normal rabbit serum, with a substance or substances in the bacterial surface causes an increase in cohesiveness, decrease in surface potential difference and characteristic alteration in wetting properties of the bacteria, and *prepares the bacteria for phagocytosis.*

2. (*a*) The effective substance or substances in the serum may become so altered as the result of heating or aging that combination with the bacterial surface, while causing changes in bacterial surface properties indistinguishable by the present physical-chemical tests from these just mentioned, *may not lead to phagocytosis*, or may lead to phagocytosis with a prezone not paralleled by a prezone in the changes in surface properties.

(*b*) Sensitization of bacteria with human sera causes changes in surface properties similar to those caused by rabbit sera, but does not lead to phagocytosis by rabbit leucocytes. The spreading requirements of rabbit polymorphonuclear leucocytes are evidently highly selective.

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