

STUDIES OF THE EFFECT OF BACTERIAL ENDOTOXINS ON RABBIT LEUCOCYTES

I. EFFECT OF INTRAVENOUS INJECTION OF THE SUBSTANCES WITH AND WITHOUT INDUCTION OF THE LOCAL SHWARTZMAN REACTION

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When bacterial endotoxins are injected intravenously into a rabbit a profound leucopenia usually develops (1). This leucopenia, primarily a granulocytopenia, is maximal within 1 to 2 hours, and 6 or 7 hours after the intravenous injection, has usually been replaced by a leucocytosis. At the time of the leucopenia, the pulmonary alveolar capillaries are found engorged with large numbers of leucocytes (2). These changes are not appreciably altered by a prior intradermal injection of endotoxin given to prepare the rabbit for the Shwartzman reaction, nor are they affected by the development of the local Shwartzman reaction, however severe. Histological sections of the skin removed 24 hours after the intradermal injection of these endotoxins show a prominent leucocytic infiltration. Histological study of the Shwartzman reaction, which usually follows the intravenous injection of endotoxin in such a prepared rabbit, reveals an even more severe leucocytic infiltration with hemorrhage. Numerous dilated, thrombosed veins and capillaries are also seen (3). The thrombi are perhaps unusual in that masses of leucocytes are included in the fibrin. It has been suggested that the thrombi are the result of agglutinated masses of leucocytes and platelets (4). The inhibition of the local Shwartzman reaction by heparin does support the concept that the local Shwartzman reaction is the consequence of a hemorrhagic infarction, but the role of the leucocyte in the thrombus formation has not been established (5, 6). It has been shown, however, that if rabbits are first treated by x-radiation, nitrogen mustard, or benzene, the Shwartzman reaction can no longer be induced although other effects of the intravenously injected bacterial endotoxins, such as pyrogenicity and lethality, remain unaltered (7, 8). The inhibitory action of nitrogen mustards and probably of benzene on the Shwartzman reaction has been correlated with their leucopenic effect, for if

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bone marrow is protected from the action of nitrogen mustard or if sulfapyridine is administered with the benzene, leucopenia fails to develop or is less severe and the Shwartzman reaction still may be induced in these animals (9, 18). It has been reported that bacterial endotoxins directly inhibit the migration of guinea pig and human leucocytes *in vitro* (10, 11).

In view of the observations which suggest that leucocytes, especially the polymorphonuclear cells, are affected by the intravenous injection of bacterial endotoxins and that the resulting leucocytic alterations may be somehow related to the development of the local Shwartzman reaction, we have used methods of tissue culture to study the effects of bacterial endotoxins upon rabbit leucocytes.

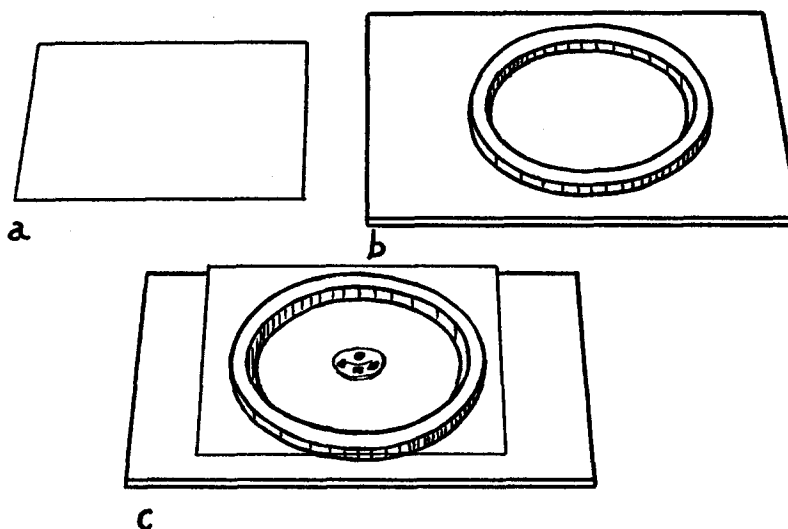
Materials and Methods

Male and female rabbits of various breeds, weighing between 2000 and 3000 gm. were used. The bacterial endotoxins used were the P-25 and P-35 polysaccharides of *Serratia marcescens* (obtained through the courtesy of Dr. M. J. Shear, National Institutes of Health, Bethesda), and a toxic antigen prepared from *Shigella flexneri* Type Z by pyridine extraction (12). Only P-25 was used in studies of the Shwartzman reaction, while all 3 toxins were used for single intravenous injections.

Animals were prepared for the local Shwartzman reaction by the intradermal injection of 50.0 μ g. (0.2 ml.) of P-25, and the reactions were provoked 24 hours after preparation by the intravenous injection of either 125.0 μ g. (0.5 ml.) or 150.0 μ g. (0.6 ml.) of P-25. All intradermal injections were made into the skin of the rabbit's flank or abdomen, the hair having been removed with electric clippers or razor. Intravenous injections were made into marginal ear veins. Of a large number of rabbits used, from 60 to 80 per cent developed positive reactions; those which did not were discarded from the study. Development of hemorrhage and necrosis at the skin site were the criteria for positive reactions. When tests were made on rabbits which received only an intravenous injection, 125.0 μ g. (0.5 ml.) or 150.0 μ g. (0.6 ml.) of P-25, 25.0 μ g. (0.25 ml.) of P-35, and 25.0 μ g. (0.25 ml.) of the *Shigella* toxic antigen were given. The criteria of effect in these animals, which had not been previously prepared for the local Shwartzman reaction, were the development of a marked fever and leucopenia.

Observations on leucocyte migration were made on tissue cultures of fragments from buffy coats of centrifuged blood, using either the glass slide method (13) or the "slide cell" technique (14). Blood was obtained from the rabbits by aseptic cardiac puncture. To prevent coagulation, all syringes and needles were coated with aqueous heparin (10 mg. per ml.) to provide a concentration of approximately 1:40,000 in the withdrawn blood. Plasma with this concentration of heparin remained fluid in test tubes, yet usually clotted promptly upon addition of tissue or leucocyte buffy coat fragments. 20 to 50 ml. of blood was withdrawn from each animal and placed into sterile glass tubes for centrifugation. Buffy coat containing leucocytes and platelets was obtained after the blood had been centrifuged at 2400 R.P.M. in the cold room at 2°C. for 20 to 30 minutes. The plasma was removed with sterile pipettes and placed in sterile glass tubes for subsequent use. If not used immediately the plasma was kept in the cold room. When plasma was not used until the following day, it was stored in the deep freeze at -25°C. during the interval. After the plasma was removed, the leucocyte layer was separated from and lifted off of the red cell layer with a sterile spatula or it was drawn into wide mouthed pipettes. This buffy coat material was placed then in Petri dishes in non-pyrogenic sterile physiological salt solution, either balanced salt solution (15) or saline. Here the white cell clump was separated from apparent bloody

fragments, divided into small pieces about 0.5 mm. square with sharp sterile surgical blades, and washed with the salt solution at least 3 times to remove plasma and red cells. From 2 to 6 pieces of buffy coat were placed immediately onto large sterile coverglasses, the excess salt solution removed, and one drop of plasma added; the fragments were washed gently in this plasma by pipetting the plasma off and on several times, finally leaving the plasma covering the fragments. The number of fragments per slide cultured averaged between 3 and 4. A large sterile glass slide on which a sterile vaseline-coated metal disc had been previously affixed by means of gentle heat was then pressed down on the coverslip so that the plasma drop which covered the tissue fragments was enclosed in a sterile air tight compartment, yet did not touch either edges or top (Text-fig. 1). These preparations were incubated at 37°C.



TEXT-FIG. 1. *a*, 30 by 35 mm. cover slip (No. 3 thickness) on which tissue fragments are placed; *b*, 3 by 1½ inch glass slide with aluminum ring attached by vaseline; *c*, completed preparation.

and were observed microscopically at 4, 6, 24, and 48 hours. Leucocyte migration extended into the clot which formed around the fragments of buffy coat. Tissue cultures made with this technique are not satisfactory if observations are extended beyond a 4 or 5 day period since cellular growth is not supported beyond this time without addition of fresh plasma or nutrient medium. Our observations were made with particular interest in the migration which occurred between 4 and 24 hours since we were primarily concerned with the polymorphonuclear leucocytes.

Tissue cultures of fragments of lung and spleen of animals killed by etherization or by the injection of air were prepared in essentially the same manner. In addition, leucocytes were obtained from the lungs and spleen by placing the aseptically removed organs in Petri dishes in which the tissue was cut with sharp knives into tiny fragments. The salt solution in which the fragments were immersed rapidly became bloody. The resulting finely divided tissue and solution were then filtered through one thickness of gauze into a 15 ml. centrifuge tube, and the fragments in the gauze further rinsed with several more milliliters of salt solution. The resulting 10 to 12 ml. of bloody fluid, now freed of tissue fragments, was then centrifuged

at 2400 R.P.M. for 20 minutes in the cold and a buffy coat could be lifted or pipetted off of the small layer of packed red cells. Fragments of this buffy coat were then cultured using the above procedure.

All cultures were made using plasma from a normal and from the test animal as the supporting media. Additional cultures were also made in both normal and Shwartzman-positive animal's plasma to which toxin was added *in vitro*. Varying concentrations of such plasma-toxin mixtures were used. Representative slides from each separate culture experiment were fixed with absolute methyl alcohol and stained with either Giemsa or Wright's stain for detailed study and photographs.

Animals were bled in the same manner when the "slide cell" technique was used. The blood was placed in vaseline-coated sterile tubes in ice water. All subsequent procedures until incubation were carried out in the cold. The "slide cells" were made in the manner described (14), but parafilm¹ was used instead of filter paper saturated in paraffin. The chamber was allowed to fill with blood from a capillary pipette and then was sealed with sterile vaseline. The slides were centrifuged at 700 R.P.M. for 10 to 15 minutes following which they were incubated in an upright position at 37°C. for 1 hour prior to observation of leucocyte migration from the buffy coat. The "slide cell" preparation for study of leucocyte migration simplifies such observations but does not allow experiments using different substrates. It does eliminate the considerable manipulation and more rapid centrifugation of the cells employed in the tissue culture technique. Regardless of the technique used, however, the results were the same, though our studies with the "slide cell" method did not include a repetition of the experiments of endotoxin influence on normal cell migration when the toxin has been added to normal *blood in vitro*.

White blood cell counts were done in the usual fashion, and differential counts were performed on smeared slides stained with Wright's method.

In all observations duplicate cultures were carried out on test and control animals in each experiment. The preparation of test and control animal leucocytes and of lung and spleen fragments was always carried out concomitantly, the planting of the test and control animal cell fragments being done in alternating sequence.

EXPERIMENTAL RESULTS

Effect of Bacterial Endotoxin on Normal Leucocytes in Vitro.—Martin and Chaudhuri have reported that bacterial endotoxins in concentrations from 0.005 to 0.58 $\mu\text{g.}$ per ml. quantitatively impaired the migration of normal human leucocytes *in vitro* in "slide-cell" preparations (11). When our preliminary observations indicated that the intravenous injection of endotoxins resulted in complete inhibition of leucocyte migration from buffy coat fragments, it became essential to restudy the action *in vitro* upon leucocytes of concentrations of endotoxin comparable to and exceeding those levels attained by the intravenous injections, using identical methods of preparation and culture.

Normal rabbits were bled and tissue cultures of buffy coat prepared in the manner described. Cultures were made of buffy coat fragments in various plasma substrates. Autologous and homologous² normal rabbit plasma were used. In other experiments, normal plasma to

¹ Marathon Corporation, Menasha, Wisconsin.

² Autologous refers to plasma obtained from the same animal. Homologous refers to plasma obtained from a different animal of the same species.

which varying amounts of endotoxin had been added was employed. It was calculated that a maximal concentration of circulating endotoxin of 1 $\mu\text{g.}$ per ml. might be reached after an intravenous injection of 150 $\mu\text{g.}$ into a 2000 gm. rabbit. Therefore, plasma-toxin mixtures were prepared to contain from 1 to 25 $\mu\text{g.}$ of P-25 per ml. of plasma. Buffy coat was planted in such plasma-toxin mixtures immediately after preparation as well as in plasma-toxin mixtures which had been incubated at 37°C. for 30 to 60 minutes. In other experiments, buffy coat fragments were first washed in salt solution containing similar concentrations of toxin to insure intimate contact of the toxin with the cells. Toxin was also added to normal rabbit whole blood which was either centrifuged immediately for buffy coat studies or incubated for 30 to 60 minutes at 37°C. before centrifugation and culture of the buffy coat. In still other experiments, the buffy coat from the blood of normal rabbits was cultured in plasma obtained from animals which had received intravenous injections of the endotoxin. This plasma was usually obtained about 2 hours after the intravenous injection of the toxin, at the time of appearance of the positive dermal reaction, but was occasionally removed 5 to 10 minutes after the intravenous toxin injection.

In none of these experiments was there any evidence that the presence of the bacterial endotoxin in the plasma substrate or blood *in vitro* inhibited the migration of normal leucocytes, when compared with the migration of leucocytes from similarly handled normal buffy coat fragments cultured in normal autologous or homologous plasma (Table I) (Figs. 1 to 3).

Effect of the Intravenous Injection of Endotoxins on Leucocytes during the Induction of the Local Shwartzman Reaction.—During the induction of the local Shwartzman reaction, a profound leucopenia occurs, as is well known (4). White blood cells accumulate in large numbers in the pulmonary capillaries, and the thrombi which form in the local skin site contain enormous numbers of leucocytes. In order to study the pathogenesis of these phenomena, leucocytes from animals which were prepared for the Shwartzman reaction and which received intravenous injections of endotoxins were studied in tissue culture.

The coverslip and the "slide cell" techniques were used. Leucocytes were obtained from rabbits at the time of the appearance of the hemorrhagic local Shwartzman reaction and were compared with leucocytes removed from control rabbits. The fragments of buffy coat from the Shwartzman-positive animal were cultured in plasma taken either from the same animal prior to the intradermal preparation and kept frozen during the interval, or in plasma from an uninjected rabbit. These fragments were also grown in the animal's own plasma obtained at the time of preparation of the buffy coat. Leucocytes were also planted in normal autologous or homologous plasma to which endotoxin had been added in varying concentrations. These fragments were then compared with identical preparations of normal rabbit buffy coat. In one experiment, leucocytes of the Shwartzman-positive rabbit were compared with the same animal's cells withdrawn immediately prior to the intravenous endotoxin injection.

Practically no migration of leucocytes was observed from the buffy coat of the Shwartzman-positive animals, in contrast to the usually excellent migration commencing promptly and continuing for at least 24 hours from cultures of normal buffy coat or from buffy coat obtained from the same animal prior

to the intravenous injection of endotoxin (Table II) (Fig. 4). Polymorphonuclear leucocytes show pyknosis, fragment, and disappear after about 24 hours in these preparations. In the normal buffy coat preparations mono-

TABLE I
Effect of Bacterial Endotoxin (P-25) in Vitro upon the Migration of Leucocytes from Normal Rabbits

A. Influence of normal plasma and plasma with added toxin on migration of normal leucocytes.

Tissue culture substrate	No. of rabbits	Leucocyte migration from explants* after 24 hrs.' incubation	
		Good	Poor
Normal plasma	5	67	2
Normal plasma with 5 μ g. toxin/ml.	1	18	0
Normal plasma with 10 μ g. toxin/ml.	5	58	6
Normal plasma with 10 μ g. toxin/ml. incubated for 1 hr. at 37°C. before adding to explants	3	24	0
Normal plasma with 25 μ g. toxin/ml.	2	34	0
Normal plasma with 25 μ g. toxin/ml. incubated for 1 hr. at 37°C. before adding to explants	1	39	0
Plasma from animal at time of appearance of positive local Shwartzman reaction	5	45	8

B. Migration of leucocytes from normal rabbit blood to which toxin had been added prior to centrifugation (10 μ g./ml.).

Time blood-toxin mixture incubated prior to centrifugation	Tissue culture substrate	No. of rabbits	Leucocyte migration from explants* after 24 hrs.' incubation	
			Good	Poor
30 min.	Autologous plasma	2	20	7
30 min.	Normal rabbit plasma	2	26	4
No incubation	Autologous plasma	1	14	0
No incubation	Normal rabbit plasma	1	16	0

* Total number of fragments of buffy coat studied in culture with approximately 3 to 4 explants per slide.

nuclear cells slowly migrate from the explant and remain alive and active during the 24 to 72 hour period of observation. In the preparations from the Shwartzman-positive animals, no migration of mononuclear cells occurred.

Time Required, after Intravenous Injection of Endotoxin, for the Development of Leucocyte Effect and Duration of Effect.—The local Shwartzman reaction is apparent to inspection 1½ to 2½ hours after an intravenous injection of endotoxin into the prepared rabbit. Leucopenia, detectable within about 20

minutes of the intravenous injection, has become profound prior to the gross appearance of the local Shwartzman reaction. It was important to learn how soon after the intravenous injection of endotoxin the inhibition of leucocyte

TABLE II
Effect of Endotoxins on Leucocytes during the Induction of the Local Shwartzman Reaction

Tissue culture substrate	Leucocyte migration, from explants* after 24 hrs. incubation					
	Shwartzman-positive rabbits			Control rabbits		
	No. of rabbits	Good	Poor to 0	No. of rabbits	Good	Poor to 0
Normal plasma	7	8	66	8	88	6
Plasma from Shwartzman positive rabbit	11	3	120	6	66	10
Normal plasma or plasma from Shwartzman-positive rabbit with added toxin (5 $\mu\text{g./ml.}$)	3	7	19	1	12	0
Normal plasma or plasma from Shwartzman-positive rabbit with added toxin (10 $\mu\text{g./ml.}$)	6	6	60	8	101	12

* Total number of fragments of buffy coat in culture with approximately 3 to 4 explants per slide.

migration could be detected and whether this inhibitory effect might antedate the development of the leucopenia.

For this purpose, cells and plasma were obtained by cardiac puncture from rabbits prepared 24 hours previously by the intradermal injection of endotoxin. Intravenous endotoxin was then given and a repeat cardiac puncture made as soon as possible. The time interval between the intravenous toxin injection and the subsequent cardiac puncture was from 5 to 20 minutes. Very slight changes in leucocyte counts had occurred in these intervals. These animals subsequently developed positive Shwartzman reactions. Fragments of buffy coat obtained from both cardiac punctures were cultured in plasma drawn from the animal before and after the toxin injection and with and without the *in vitro* addition of endotoxin to each.

Regardless of the plasma substrate used, there was very slight to no migration of leucocytes from the fragments of buffy coat obtained after the intravenous toxin injection in contrast to the excellent migration of cells from the buffy coat of the same animals prior to the intravenous injection (Table III) (Figs. 5 and 6).

A buffy coat was obtained from an animal 6 hours after the intravenous toxin injection and 4 hours after the development of a positive Shwartzman reaction. The cultures of buffy coat showed markedly impaired migration of leucocytes when compared to normal leucocytes, but some migration was seen. Very little difference could be noted between the rate and extent of leucocyte migration from the buffy coat obtained 12 hours after intravenous

TABLE III

Time Interval Required after the Intravenous Injection of Endotoxin for Development of the Leucocyte Effect in the Shwartzman-Reacting Animal

Time cells obtained (same rabbit)	Tissue culture substrate	Leucocyte migration from explants* after 24 hrs. incubation	
		Good	Poor to 0
5 min. before intravenous toxin injection	Plasma before intravenous toxin injection	36	2
	Plasma 5 min. after intravenous toxin injection	39	3
5 min. after intravenous toxin injection	Plasma before intravenous toxin injection	0	25
	Plasma 5 min. after intravenous toxin injection	0	30

* Total number of fragments of buffy coat studied in culture with approximately 3 to 4 explants per slide.

TABLE IV

Duration of the Inhibitory Effect on Leucocyte Migration of Endotoxin in the Shwartzman-Reacting Animal

Time cells obtained	No. of rabbits	Tissue culture substrate	Leucocyte migration from explants* after 24 hrs. incubation		
			Good	Fair	Poor to 0
6 hrs. after intravenous toxin injection (4 hrs. after + Shwartzman)	1	Normal plasma	0	14	22
		Plasma 6 hrs. after intravenous toxin injection	0	0	26
13 hrs. after intravenous toxin injection (11 hrs. after + Shwartzman)	1	Normal plasma	16	2	0
		Plasma 13 hrs. after intravenous toxin injection	22	3	0

* Total number of fragments of buffy coat studied in culture with approximately 3 to 4 explants per slide.

injection, 11 hours after the positive reaction had appeared, and the rate and extent of migration from normal buffy coat (Table IV).

It appears, therefore, that the change in the leucocytes occurs within 5 minutes after the intravenous injection of toxin and lasts for at least 6 hours (Figs. 6 to 8).

Effect of the Preparation of the Skin for the Shwartzman Reaction on Leucocyte

Migration in Vitro.—The most specific and perplexing characteristic of the local Shwartzman reaction is the effect of the preparation of the skin. While a number of substances other than bacterial endotoxins will induce the reaction when injected intravenously into the prepared rabbit, it appears that only bacterial endotoxins will properly prepare the skin for the reaction. Other substances, which may result in equal inflammation upon intradermal injection, will not prepare the skin for the reaction. It seemed of interest to see whether preparation of the skin with endotoxin rendered the leucocytes more susceptible, at the end of 24 hours, to the effect of the toxins *in vitro*.

For this purpose several rabbits were prepared in the usual manner with bacterial endotoxin. 24 hours after the intradermal injection and just prior to the intravenous injection, all were bled from the heart as was a normal animal. Plasma and buffy coat were prepared and stored at 2°C. When Shwartzman reactions were apparent, buffy coat from a reactor was chosen for culturing as well as that from the control.

Leucocytes migrated equally from the buffy coat fragments of the Shwartzman-prepared animal in normal plasma, plasma removed from the animal after intravenous injection of toxin at the time of the development of the local reaction, and normal plasma to which 5 μ g. of toxin per ml. had been added, as well as did leucocytes from the buffy coat of the control. No leucocyte migration in any substrate occurred from the buffy coat which was obtained after the intravenous injection of toxin at the time of the development of the local reaction.

It would thus appear that prior preparation of the skin with toxin for the Shwartzman reaction does not render the leucocytes susceptible to endotoxin *in vitro* when the leucocytes are obtained at the time of the usual intravenous provocative injection 24 hours after skin preparation.

Effect of Heparin on the Leucocyte Inhibitory Action of Endotoxins.—Since heparin has been found to inhibit the local Shwartzman reaction (5, 6), and since the agglutination of leucocytes has been considered as a possible cause of the thrombi seen in vessels at the site of the positive local reaction, it was important to determine the effect of heparinization upon the action of the intravenously injected endotoxins on the leucocyte.

Buffy coats from several animals previously found to develop a positive Shwartzman reaction, and in which the reaction was now inhibited by the administration of 5 mg. of heparin every 30 minutes, were obtained 2 hours after the intravenous injection of endotoxin. Tissue cultures were made and no migration of leucocytes occurred in either autologous or homologous normal plasma.

While heparin inhibited the local Shwartzman reaction it did not prevent the development of leucopenia (6) nor did it protect the leucocyte from the inhibition of migration occurring as a result of the intravenous injection of endotoxin.

Effect of Intravenous Endotoxin on the Leucocytes in the Lung and Spleen.—With the development of the local Shwartzman reaction, leucocytes have been observed in large numbers in the capillaries of the lungs. Presumably these cells accumulated in the lungs either as a result of alterations of the leucocytes or of changes in the pulmonary vascular bed. Evidence so far presented suggested that the intravenous injection of endotoxins does alter the leucocyte as measured by migration from buffy coat fragments. It was desirable to learn how the leucocytes, which had accumulated in the lung and spleen during

TABLE V
Migration of Leucocytes from Fragments of Lung and Spleen of Rabbits at Time of Development of Local Shwartzman Reaction

Tissue	No. of rabbits	Tissue culture substrate	Leucocyte migration from explants* after 24 hrs. incubation		
			Good	Fair	Poor to 0
Normal lung	2	Normal plasma	0	20 (48 hrs.)	20 (24 hrs.)
Lung from Shwartzman-positive rabbit	4	Autologous plasma	108	31	9
Normal spleen	2	Normal plasma	48	20	0
Spleen from Shwartzman-positive rabbit	4	Normal plasma	25	2	3
		Autologous plasma	120	27	7
		Normal or autologous plasma with added toxin	78	18	8

* Total number of fragments of tissue studied in culture with approximately 3 to 4 explants per slide.

the production of the local Shwartzman reaction, would behave in tissue culture.

Fragments of sterile lung were prepared in essentially the same manner as the fragments of buffy coat, but the lung tissue was not centrifuged. Tissue cultures of fragments of normal rabbit lung showed migration of only a few polymorphonuclear leucocytes during the first 24 hours, at which time large mononuclear cells appeared in small numbers.

The culture of uncentrifuged fragments of lung obtained from animals 2 hours after intravenous injection of toxin and at the time of the appearance of the Shwartzman reaction, showed a prompt and massive migration of leucocytes, surpassing that usually seen from normal buffy coat. This migration occurred in the reacting animal's plasma as well as in normal plasma (Table V) (Figs. 9 to 11).

When normal spleen was cultured in plasma, there was a prompt migration of cells which, for the first 12 hours, were largely polymorphonuclear leucocytes. Monocytic cells migrated into the plasma clot more slowly, but after 24 hours became predominant.

Portions of spleen were obtained from normal animals as well as from animals 2 hours after the intravenous toxin injection at which time positive Shwartzman reactions were present. Fragments were prepared for culture in essentially the same manner as the preparation of buffy coat but were not centrifuged.

It was found in a number of experiments that the migration of both polymorphonuclear and mononuclear cells was approximately equal from the fragments of spleen of the Shwartzman-reacting and the control animals in normal plasma, in plasma from the reacting animal, and in both to which toxin had been added *in vitro* (Table V) (Figs. 13 to 15). While the cells from the spleen of the Shwartzman-positive animal perhaps showed slightly less migration and seemed to succumb earlier, the differences were not considered significant in these experiments.

Effect of Centrifugation upon the Migration of Leucocytes from Animals Receiving Bacterial Endotoxins.—When it was found that the leucocytes in the spleen and lung of an animal receiving intravenous endotoxin migrated well from tissue fragments but not from the buffy coat of the same animal, it became essential to study the effect of centrifugation on leucocytes which had been exposed to the endotoxin *in vivo*.

Warm stage examination was made of the whole blood of rabbits drawn 2 hours after receiving intravenous injections of endotoxins at the time of the development of a positive Shwartzman reaction. Buffy coat was obtained from the same animal and resuspension of cells of such buffy coat fragments in saline attempted.

It was found that polymorphonuclear leucocytes in the whole blood from animals receiving endotoxin, while located with difficulty because of the leucopenia, exhibited motility that was in every way as rapid and persistent as that of polymorphonuclear cells from normal blood. Cells from dispersed buffy coat, however, for the most part failed to migrate on the warm stage. It must be noted that it was very difficult to break up these buffy coat fragments which had been obtained at speeds of 2400 R.P.M. and cells often remained in tiny clumps in spite of considerable agitation in warm saline. An occasional leucocyte from such buffy coat resuspensions was seen to migrate normally on the warm stage. When blood from rabbits which received intravenous endotoxin was collected at 2 to 5 hours after injection and was centrifuged at 700 R.P.M., the buffy coat could be resuspended with relative ease. Warm stage preparations of the blood before centrifugation or after resuspending the buffy coat by shaking showed migration of individual leucocytes comparable to that of normal controls treated in the identical manner

even though buffy coat preparations from the blood of the injected animal centrifuged at 700 R.P.M. failed to show leucocyte migration in tissue cultures or in the "slide cell."

Both lungs were removed from rabbits using precautions for asepsis. These rabbits had been previously prepared for the local Shwartzman reaction. They had been injected with an intravenous provocative dose of 150 μ g. of endotoxin and, with the development of a positive local reaction, were operated upon. The lungs were cut up into fine fragments in salt solution, and the resulting bloody solution filtered through gauze; some of the solution was saved for warm stage preparations and the rest was centrifuged at 2400 R.P.M. in the cold. Fragments of lung tissue were also saved for culture without centrifugation.

Buffy coat material obtained by centrifuging saline washings of fragments of lungs of animals receiving intravenous endotoxin showed markedly im-

TABLE VI
Effect of Centrifugation upon the Migration of Leucocytes from the Lung and Spleen Tissue of Shwartzman-Positive Animals

Tissue	No. of rabbits	Tissue culture substrate	Leucocyte migration from explants* after 24 hrs. incubation		
			Good	Fair	Poor to 0
Buffy coat fragments from saline washings of lung	3	Autologous plasma	13	24	95
Buffy coat fragments from saline washings of spleen	2	Autologous plasma	0	8	24

* Total number of fragments of buffy coat studied in culture with approximately 3 to 4 explants per slide.

paired migration in tissue cultures in spite of the fact that uncentrifuged fragments of the same lung showed excellent leucocytic migration in otherwise identically prepared cultures (Table VI) (Fig. 12). In one experiment, the white blood cell count of the saline washings prior to centrifugation was 4050 with 25 per cent polymorphonuclear cells. Leucocytes obtained by such saline washings of the lung migrated normally, however, on the warm stage prior to centrifugation.

The whole spleens from animals developing a positive local Shwartzman reaction were removed aseptically. Each spleen was cut up in saline and the washings filtered through gauze; some of the washings was saved for warm stage preparations and the rest was centrifuged in the cold at 2400 R.P.M. Fragments of spleen were also saved for culture.

There was a markedly impaired migration of cells from the buffy coat obtained from saline washings of fragments of spleen from Shwartzman-positive animals 2 hours after the intravenous injection of the endotoxin (Table VI) (Fig. 16). Leucocytes obtained in this manner migrated normally on the warm stage prior to centrifugation. Cell washings from normal rabbit

spleen reveal a very low proportion of polymorphonuclear cells. Buffy coat fragments of such washings, prepared by centrifugation at 2400 R.P.M., show prompt and excellent migration of leucocytes.

It is thus seen that centrifugation had altered the leucocytes from animals receiving an intravenous injection of endotoxin so that migration from the buffy coat did not occur even though these leucocytes prior to centrifugation move normally on the warm stage. Identical centrifugation did not alter normal leucocytes.

Effect of Single Injections of Bacterial Endotoxins on Leucocyte Migration in Tissue Culture.—The single intravenous injection of bacterial endotoxins produces most if not all of the systemic effects seen during the induction of the local Shwartzman reaction. Leucopenia is as profound in the rabbit receiving a single intravenous injection as in the animal suffering a severe local Shwartzman reaction. Evidence already presented indicates that the preparation of the skin has not, 24 hours later, resulted in any alteration of the migration of leucocytes in tissue culture. Nevertheless, it was important to demonstrate the effect on the migration of leucocytes of a single intravenous injection of endotoxin into unprepared rabbits.

Observations were made on the leucocytes removed from rabbits at intervals of 5 minutes, 2 hours, 5 hours, 12 hours, and 24 hours after the intravenous injection of endotoxin. Three endotoxin preparations were employed; the results with all were the same and will be reported together. All studies were initially done using the tissue culture technique, but have been repeated using the "slide cell" method. Normal control cultures were always made concomitantly for comparison. The buffy coat fragments of control rabbits and of rabbits receiving the intravenous injection of toxin were cultured in normal plasma and in plasma from the animals receiving the injections of endotoxin. As with the previous experiments on the Shwartzman-positive animals, the type of plasma substrate used did not alter the migration of the leucocytes under study.

The buffy coat of blood removed 5 minutes after the intravenous injection of toxin showed unequivocal impairment of leucocyte migration although some migration persisted. Although the rabbits at this time had no fever, slight leucopenia had developed and the white blood counts averaged 5000 per ml. At 2 hours after the toxin administration, virtually no migration occurred from the buffy coat fragments. Leucopenia was severe at this time, the count averaging 2000 cells per ml. At 5 hours after the intravenous injection some migration from the buffy coat occurred. Leucocytosis was now the rule, the count ranging between 10,000 and 20,000 cells per ml. After 12 hours, migration was good but still less than normal, while at 24 hours, no differences could be detected between the migration of leucocytes from the normal animals and from the animals receiving endotoxin (Table VII).

Tissue cultures made of spleen removed from animals killed 2 hours after a single intravenous toxin administration showed migration comparable to that

from spleen fragments from normal animals. The lung fragments from animals receiving the toxin, however, exhibited greater numbers of actively migrating leucocytes than the lung fragments from normal rabbits.

It was thus seen that a single intravenous injection of bacterial endotoxin, without prior preparation of the rabbit by intradermal injection, resulted in an inhibition of motility of leucocytes in tissue culture of the same degree and duration as that seen in the animals exhibiting a positive local Shwartzman reaction.

TABLE VII

Leucocyte Migration at Different Time Intervals after Injection of Endotoxin as Compared with Normal Controls

Toxin	No. of rabbits	Time interval	Migration	No. of cultures
P-25	2	5 min.	Fair	16/48*
	3	2 hrs.	None	22/60
	2	5 hrs.	None to slight	14/28
	2	12 hrs.	Fair	16/46
P-35	16	5 hrs.	None to slight	66
<i>Shigella</i>	12	5 hrs.	None to slight	60

* Number of individual slides/number of fragments cultured.

DISCUSSION

The mechanism of the development of the profound leucopenia which occurs in the rabbit following the intravenous injection of bacterial endotoxins has always been obscure. The observation that the pulmonary capillaries are filled with clumps of leucocytes suggests that some alteration of the leucocytes or of the vascular endothelium of the lung may be responsible for the accumulation of the leucocytes in the lung vessels. It has been suggested that an alteration of the leucocytes or of the blood vessels results in the formation of the leucocyte-rich thrombi in capillaries and venules at the site of the positive Shwartzman reaction (4). It has not been demonstrated that the leucocytes themselves are responsible for the development of those thrombi, but the thrombi have been regarded as a primary cause of the hemorrhagic necrosis which is characteristic of the local Shwartzman reaction (4). The possible relationship of the leucocytes to the thrombus formation is suggested by the failure of thrombi to form, and of the local Shwartzman reaction to develop, in the animal rendered leucopenic (9). Methods used to produce leucopenia, however, doubtlessly have altered the blood platelets and possibly other factors of blood coagulation. This aspect must be further studied. The relationship of the thrombi to the occurrence of the local Shwartzman lesion is suggested by the inhibition of the reaction by heparin (5, 6).

Our experiments indicate that an intravenous injection of bacterial endotoxins into rabbits can alter the circulating leucocytes in such a way that they fail to migrate out of the buffy coat, even though leucocytes from the same animal prior to endotoxin injection do so under identical circumstances of centrifugation and tissue culture. This effect is evident, but is not complete, within 5 minutes after the injection, and persists for as long as 6 to 12 hours. It is the same in animals previously prepared for, and developing, a severe local Shwartzman reaction, as in those receiving a single intravenous injection of the endotoxin.

The inhibitory effect upon the motility of the leucocytes must depend upon some action of the toxin *in vivo*, for the motility of rabbit leucocytes is unaltered by large concentrations of the endotoxin *in vitro*, whether added before or after centrifugation, according to our technique. Also the inhibitory effect is not a result of the release of any "toxic" substance from tissue cells into the blood stream; for normal leucocytes migrated well in the plasma from animals which had received intravenous endotoxin from 5 minutes to 2 hours before bleeding.

The inhibition *in vitro* of normal leucocyte migration by these bacterial endotoxins, as reported by others, (10, 11) stands in contrast to our observations. Morgan (10) noted that guinea pig leucocytes failed to migrate from splenic explants into capillary tubes containing toxic somatic antigen of *S. typhosa* thus showing a negative chemotaxis of this material. Martin and Chaudhuri (11) showed that various concentrations of typhoid somatic antigen, meningococcus filtrate, and the P-25 of *S. marcescens* quantitatively inhibited the migration of human leucocytes in "slide cell" preparations. They observed considerable variation in the sensitivity of leucocytes from different individuals. Quantitative measurements of leucocyte migration in the "slide cell" technique may not be reliable. Our experiences with large numbers of cultures with coverslip tissue explants indicate that rabbit leucocytes migrate as well in substrates having concentrations of endotoxin many times greater than those employed by Martin and Chaudhuri as in normal plasma. Preliminary observations have disclosed good migration of human leucocytes from buffy coat fragments obtained after centrifugation of blood for 20 minutes at 2400 R.P.M. grown in plasma substrates containing 25 μ g. of endotoxin per ml.

The action of the endotoxin upon the leucocytes requires centrifugation, if only at low speeds, for demonstration; the same leucocytes prior to centrifugation migrate normally in warm stage preparations. When centrifugation is carried out at 700 R.P.M., resuspension of the leucocytes is possible and the isolated cells again migrate normally on the warm stage even though they fail to do so from buffy coat obtained at the same speed. Cells from buffy coats obtained at 2400 R.P.M. cannot be well resuspended for adequate warm stage

examination of isolated leucocytes. Observations made thus far suggest that the intravenous injection of the endotoxin does not result in such fragility or injury that centrifugation, at least at 700 R.P.M., leads to irreversible damage. While the nature of this cell change, such as an increased cohesiveness (4) or some subtle metabolic change, remains to be clarified, it may result in the accumulation of leucocytes in the capillaries of the lungs and perhaps in the thrombi of the local Shwartzman reaction. The migration of large numbers of leucocytes from lung fragments of animals receiving the intravenous endotoxin when compared with the very scanty migration of cells from normal lung confirms the histological observation that leucocytes appear in large numbers in the alveolar capillaries of such injected animals. Cells isolated from the lung of injected animals and centrifuged failed to migrate from the buffy coat.

The action of heparin in the inhibition of the local Shwartzman reaction does not seem to depend upon this alteration of the leucocyte since the white blood cells from rabbits in which the Shwartzman reaction has been inhibited by heparin also fail to migrate from buffy coat fragments. It is of interest that substances other than bacterial endotoxins, such as agar, glycogen, and a foreign protein in the sensitized animal are capable of inducing severe leucopenia when injected intravenously, provoke the local Shwartzman reaction in the properly prepared rabbit (3, 4, 16) and, in preliminary studies, induce changes in leucocyte migration similar to the action of bacterial endotoxins (17).

SUMMARY

Intravenous injection into rabbits of bacterial endotoxins results in an inhibition of migration of leucocytes from the buffy coat of their blood in tissue culture or in "slide cell" preparations. This effect was demonstrable 5 minutes after the intravenous injection and persisted for from 6 to 12 hours after the injection. It is as marked in rabbits receiving only a single intravenous injection of endotoxin as in those previously prepared intradermally and developing a severe local Shwartzman reaction on intravenous injection. The preparation of the skin for the Shwartzman reaction does not in itself result in appreciable changes of leucocyte migration. The production of the effect depends upon some action *in vivo*, since leucocytes of uninjected rabbits migrate normally from the buffy coat in plasma substrates to which large concentrations of endotoxin are added *in vitro*. The inhibitory effect, as observed in these experiments, also depends upon the added influence of centrifugation. Leucocytes from a rabbit receiving endotoxin intravenously migrate normally from uncentrifuged lung or spleen fragments and migrate normally in blood on the warm stage prior to centrifugation. Identical centrifugation does not affect leucocytes from uninjected animals. The heparin

inhibition of the local Shwartzman reaction does not alter this effect of endotoxins on leucocytes. Its possible role in the production of leucopenia and of the local Shwartzman reaction is briefly discussed.

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EXPLANATION OF PLATES

Photomicrographs were made by Mrs. Joan Bailey.

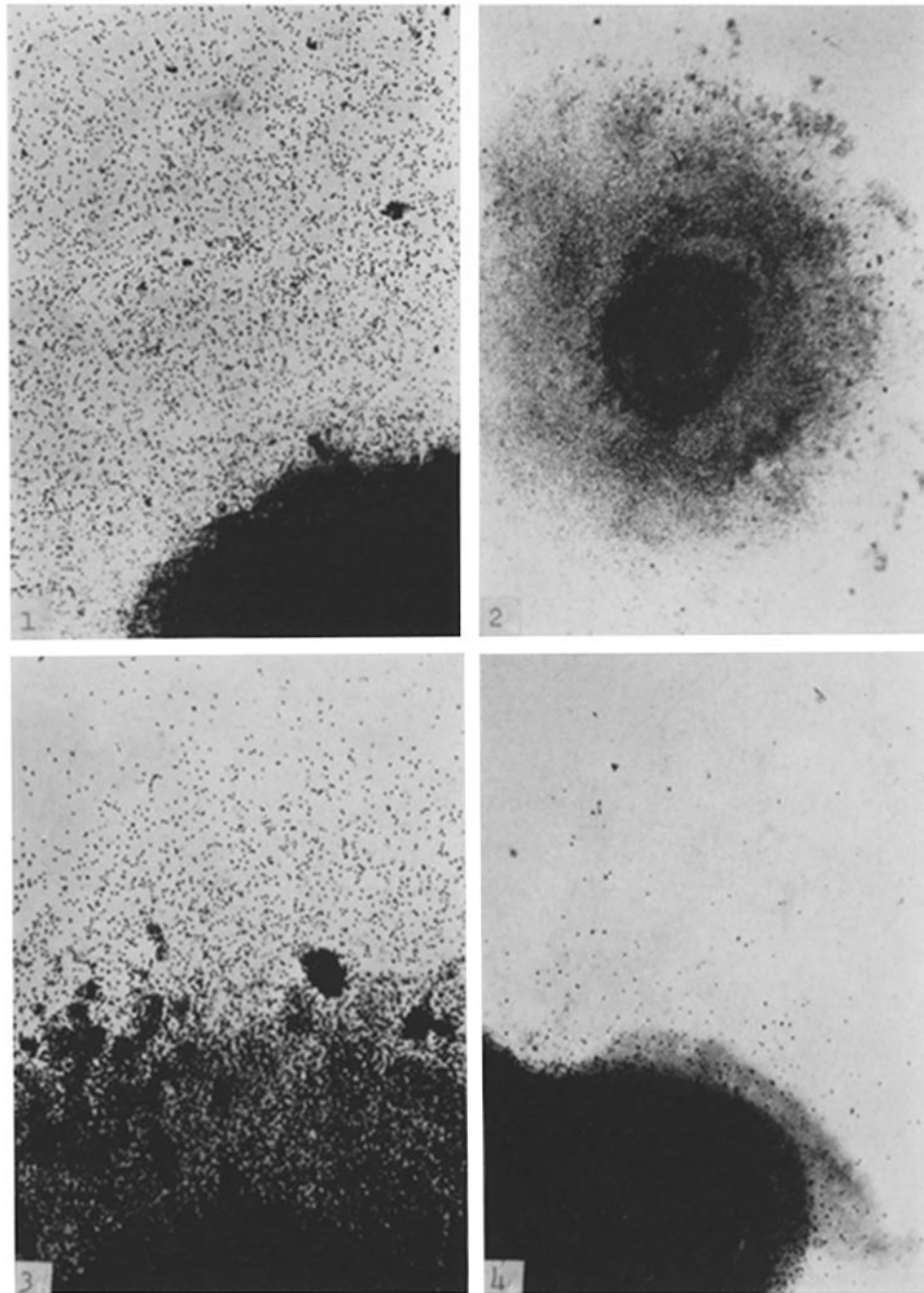
PLATE 40

FIG. 1. Leucocyte migration from buffy coat of a normal rabbit in normal plasma after 20 hours' incubation. $\times 81$.

FIG. 2. Leucocyte migration from buffy coat fragment of normal rabbit in normal plasma containing 10 μg . of P-25 endotoxin per ml. of plasma after 20 hours' incubation. Taken at low magnification to show typical tissue culture explant. $\times 23$.

FIG. 3. Same as Fig. 2 after only 6 hours of incubation. $\times 81$.

FIG. 4. Failure of leucocyte migration after 20 hours of incubation from buffy coat obtained 2 hours after endotoxin injected intravenously and at time of appearance of local Schwartzman reaction. $\times 81$.



(Berthrong and Cluff: Effect of endotoxins on leucocytes. 1)

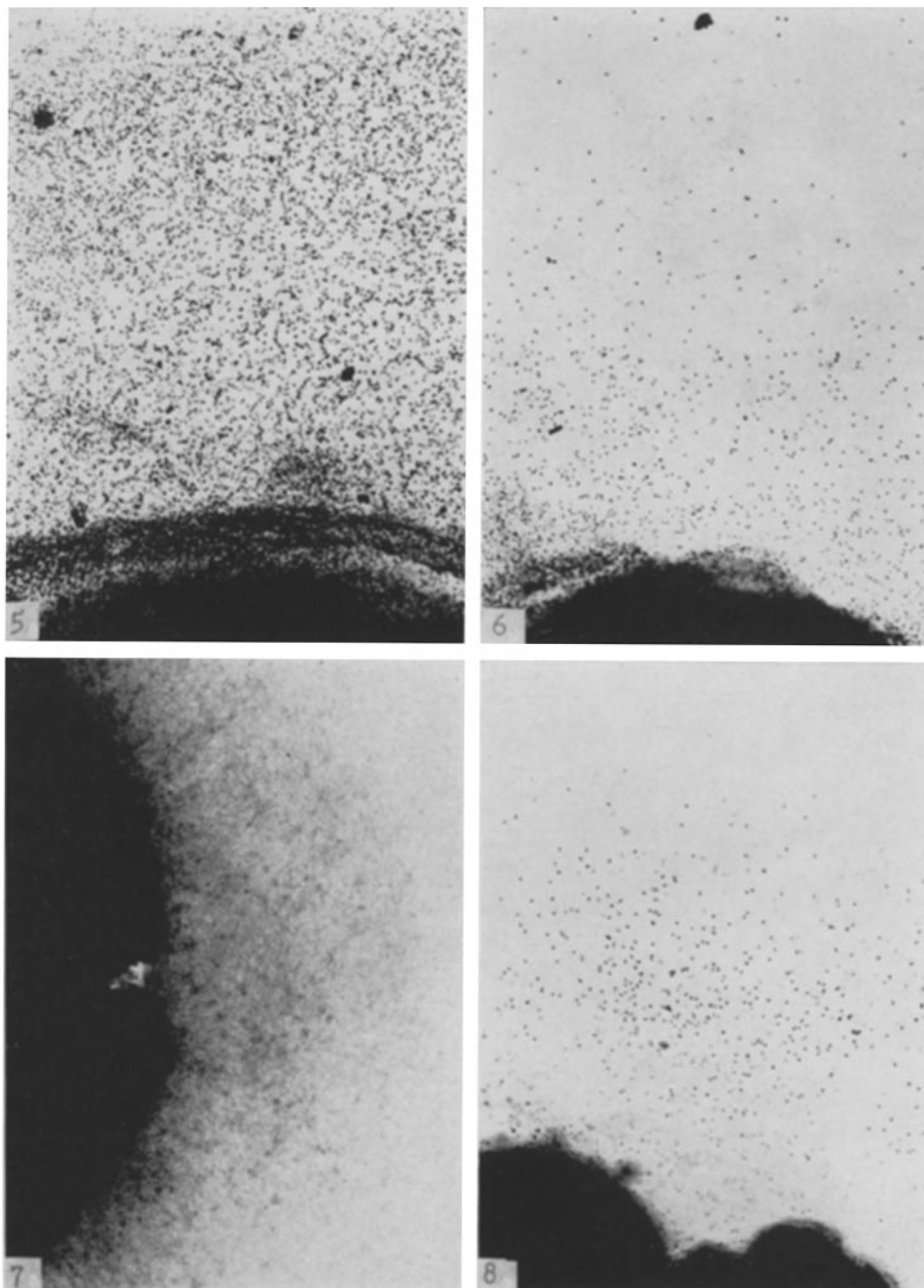
PLATE 41

FIG. 5. Leucocyte migration from buffy coat of animal 24 hours after intradermal preparation and just before intravenous injection. 20 hours' incubation in normal plasma. $\times 79$.

FIG. 6. Migration from buffy coat of same animal as Fig. 5, obtained 20 minutes after intravenous injection of endotoxin. 20 hours of incubation in normal plasma. $\times 79$.

FIG. 7. Absence of any migration of leucocytes from buffy coat fragment of same animal 2 hours after intravenous injection of endotoxin. 20 hours' incubation in normal plasma. $\times 79$.

FIG. 8. Migration of leucocytes from another rabbit 6 hours after intravenous injection of endotoxin and 4 hours after appearance of local Shwartzman reaction. 20 hours of incubation in normal plasma. $\times 79$.



(Berthrong and Cluff: Effect of endotoxins on leucocytes. I)

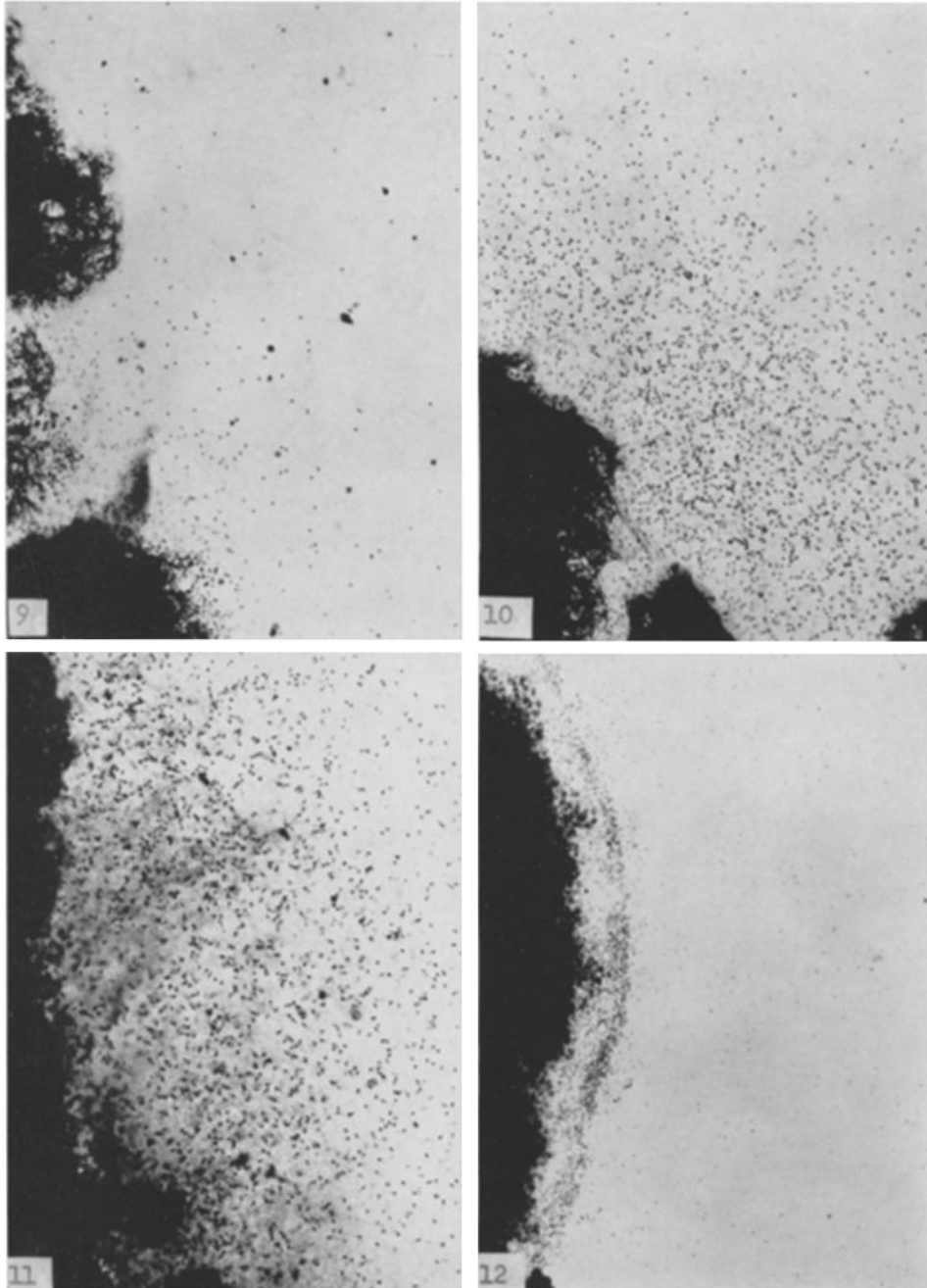
PLATE 42

FIG. 9. Leucocyte migration from lung fragments from normal rabbit. 20 hours of incubation in normal plasma. $\times 79$.

FIG. 10. Migration of leucocytes from uncentrifuged lung fragments from animal 2 hours after intravenous injection of endotoxin and at appearance of local Shwartzman reaction. 6 hours of incubation in normal plasma. $\times 79$.

FIG. 11. Same as Fig. 10 after 20 hours' incubation with slightly greater magnification. $\times 86$.

FIG. 12. Failure of migration of leucocytes from buffy coat fragments of centrifuged cell washings of lung from rabbit receiving intravenous injection of endotoxin. 20 hours of incubation in normal plasma. $\times 79$.



(Berthrong and Cluff: Effect of endotoxins on leucocytes. I)

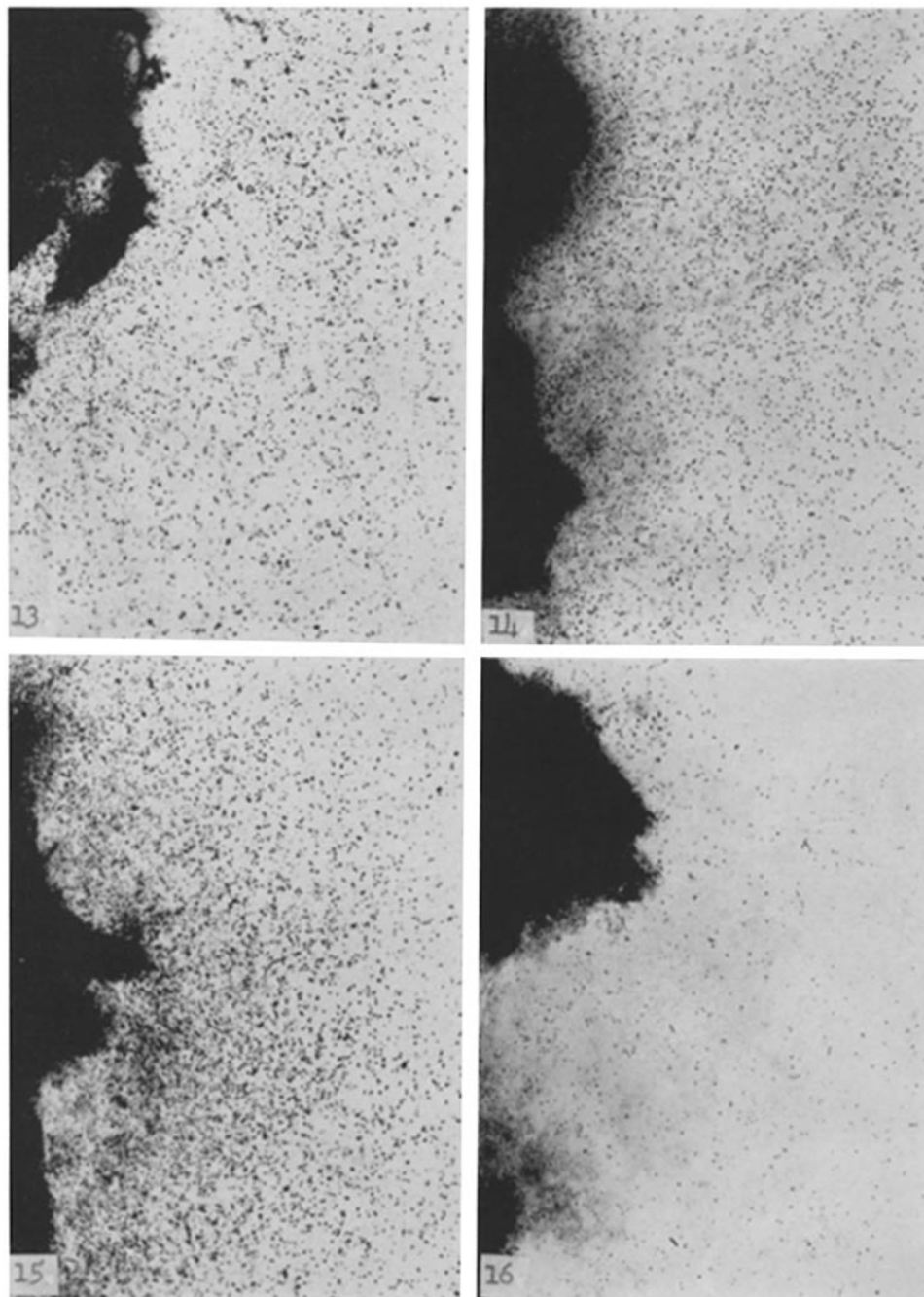
PLATE 43

FIG. 13. Leucocyte migration from fragment of spleen from normal rabbit. 18 hours' incubation in normal plasma. $\times 79$.

FIG. 14. Leucocyte migration from spleen uncentrifuged fragment from rabbit receiving intravenous injection of endotoxin. 18 hours' incubation in normal plasma. $\times 79$.

FIG. 15. Leucocyte migration from same animal as Fig. 14 after 18 hours' incubation in normal plasma containing $10 \mu\text{g}$. injection of endotoxin per ml. plasma. $\times 79$.

FIG. 16. Very poor leucocyte migration from buffy coat from centrifuged washings of spleen obtained from rabbit receiving intravenous injection of endotoxin. $\times 79$.



(Berthrong and Cluff: Effect of endotoxins on leucocytes. I)