STUDIES OF THE EFFECT OF BACTERIAL ENDOTOXINS ON RABBIT LEUCOCYTES

II. Development of Acquired Resistance

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Most Gram-negative bacteria contain a cellular component which is toxic to animals. The biological activities of these endotoxins are remarkably similar irrespective of the species of organism from which they are derived (1). In the rabbit they induce considerable fever, diarrhea, dyspnea, conjunctival hyperemia, transient thrombocytopenia, and leucopenia, and occasionally death. They can serve to prepare rabbit skin for the Shwartzman reaction and can provoke the reaction upon intravenous injection into prepared animals.

Among the characteristic histopathological findings occurring in rabbits injected with endotoxins are hemorrhagic and thrombotic phenomena. The thrombi involve capillaries and small veins and are composed of leucocytes, platelets, and a fibrin-like material. For some time it has been thought that injury to vascular endothelium by the endotoxins is responsible for the thrombotic and hemorrhagic reaction. However, recent studies indicate that the leucocyte may play a fundamental role in this relation (2, 3). A direct indication of the effect of endotoxin on rabbit leucocytes is provided by the fact that the migration of centrifuged leucocytes is impaired in blood obtained from animals that have received toxin intravenously (4).

Antiserum from animals immunized with endotoxin has very little capacity to neutralize the various effects of the toxin; such antiserum will not protect a rabbit from the Shwartzman reaction or prevent fever due to injection of the homologous toxin (5–8, 15); and only partial neutralization of the lethal action of the toxin can be achieved with antiserum (9–13). On the other hand, animals given repetitive injections of endotoxins develop resistance to fever and the Shwartzman reaction (6–8, 14–17). This resistance is of short duration, non-specific, and can be abolished by injection of thorotrast or trypan blue. Serum from resistant animals confers no protection on recipient animals. In the present study this non-specific resistance to bacterial endo-

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toxins has been investigated with respect to the effect on the migratory capacity of rabbit leucocytes.

Materials and Methods

Male and female hybrid rabbits of varying color, weighing 2000 to 3000 gm. have been used. The animals' diet consisted of water *ad lib* and Purina rabbit pellets.

A toxic antigen was obtained by pyridine extraction from *Shigella flexneri* Type Z (18). This was stored in a lyophilized state and solutions were prepared with non-pyrogenic saline so that 25.0 μg. was contained in 0.25 ml. Dr. M. J. Shear kindly supplied a toxic polysaccharide, called P-35, obtained from *Serratia marcescens*. This was kept at 4°C. in concentrated solution and diluted with saline so that 25.0 μg. was contained in 0.25 ml. Injections of toxin were given intravenously.

Blood for studies of leucocyte migration was removed from rabbits by cardiac puncture. 16 gauge needles were used to prevent excessive turbulence on withdrawing blood. An aqueous salt of heparin (Lederle) containing 10.0 mg. per ml. was used as an anticoagulant. Somewhat less than 0.05 ml. of the heparin solution was added to the syringe prior to drawing 10.0 ml. of blood, and was used to coat the surfaces with which the blood would come in contact. The blood was immediately expelled into iced tubes and all subsequent handling until incubation was done at 4°C. Sterile, petrolatum-coated tubes were usually employed, but no detectable difference was noted in migration of normal cells when blood was collected in sterile glass tubes not coated with petrolatum. The elapsed time between withdrawal of blood and beginning of incubation was usually no more, and often less, than 45 minutes.

Leucocyte migration was studied by the "slide cell" technique described by Martin, Pierce, Middlebrook, and Dubos (19). The procedure was modified by using parafilm instead of paraffin-saturated filter paper in the preparation of the slide. This modification not only simplified the method but facilitated the consistent production of well sealed preparations. The "slide cells" were placed in an 80°C. oven for 30 to 60 minutes to allow a seal to occur between coverglass, parafilm, and glass slide. The completed "slide cells" were chilled at 4°C. prior to use. The blood was allowed to fill the slide chamber by capillary action from a cold capillary pipette. Centrifugation was carried out at 600 to 700 R.P.M. for 10 to 15 minutes in the cold in an International SB2 centrifuge with a horizontal head. This consistently gave preparations with clearly discernible leucocyte-platelet layer and clarified plasma. The slides were then promptly placed at 37°C. in an upright position. Migration of leucocytes into the plasma was studied after 1 hour at this temperature. For each animal during a single observation no less than 3 and often as many as 8 replicate slides were made.

Rabbit temperatures were taken *per rectum* with a clinical centigrade thermometer. Temperatures were taken prior to injection of toxin, as control, and every hour thereafter for 6 hours. The fever curve thus obtained was plotted on graph paper with 5 by 5 squares to the square inch. An expression of the degree and duration of fever as a numerical value was obtained by measuring the area under the curve with a Dietzgen-Coradi planimeter. This value is defined as the fever index, in the manner described by Beeson (15). When indices were to be compared, all readings were made at one time to avoid differences which might exist from resetting of the planimeter.

Blood for serological tests was usually obtained from marginal ear veins. The separated serum was tested semiquantitatively for precipitating antibody. Serial twofold dilutions of the antigen were mixed with equal volumes of undiluted serum in capillary tubes. These were incubated at 37°C. for 2 hours and then refrigerated overnight before final reading. Determination of the zone of optimum precipitation was made by evaluating the rapidity of precipitation and amount of precipitate at 24 hours. Four plus was used to represent maximum precipitation.

White blood cell counts were done in the usual manner in a hemocytometer chamber. Differential leucocyte counts were made from smeared slides stained by Wright's method.

The thorotrast (Heyden) used was a 25 per cent colloidal solution of thorium dioxide.

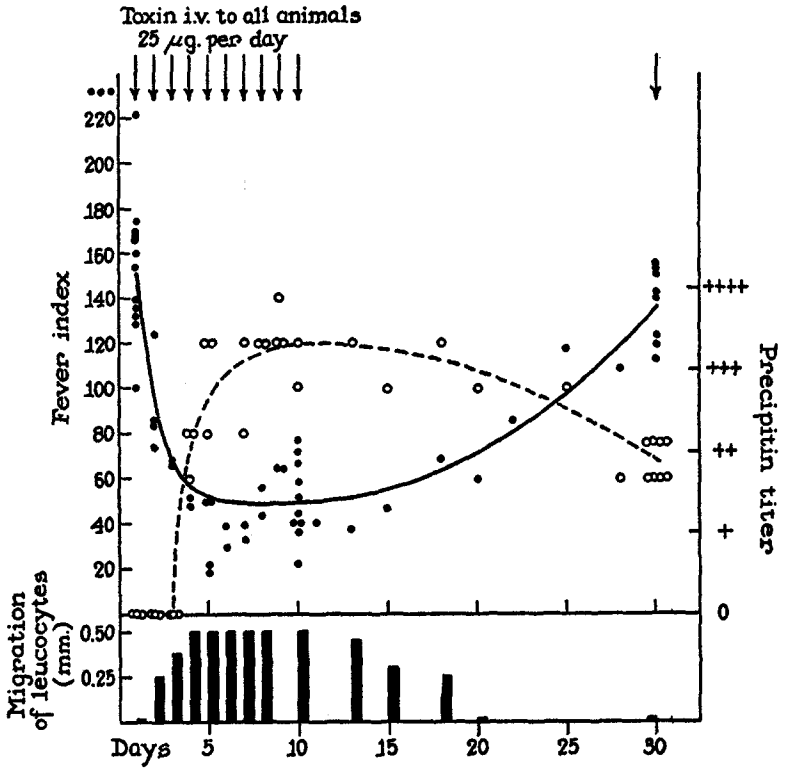
EXPERIMENTAL RESULTS

Effect of Repetitive Injections of Endotoxin on Leucocyte Migration, Febrile Response, and Precipitins.—Daily injection of bacterial endotoxin into rabbits results in development of resistance to the pyrogenic effect of the toxin (16). As mentioned previously, this resistance does not appear to be related to antibody production. Experiments were done to determine whether rabbit leucocytes develop resistance to the inhibitory effect of injected endotoxin on migration of centrifuged cells (4). The effects observed were correlated with data on the antibody titer and resistance to fever.

Experiment 1.—Thirteen rabbits were given 25.0 μg. *Shigella* toxic antigen intravenously every day for 10 days. All were tested for febrile response and leucocyte migration on the 1st day. Blood for evaluation of leucocyte migration was drawn 5 hours after injection of toxin and compared with the blood of a normal untreated animal. Daily observations were made on 4 of the 13 rabbits during the 10 days of repetitive toxin administration to determine changes in the febrile response, precipitin titer, leucocyte migration, and degree of leucopenia. Blood for precipitin analysis was obtained prior to each injection of toxin. White blood cell counts were done prior to toxin injection, and 1 and 5 hours thereafter. On the 10th day all animals were tested in this manner. During the days following the cessation of repetitive toxin injections single animals were selected at intervals of a day or two for retesting with toxin. Each of these animals was studied for leucocyte migration, febrile response, and precipitating antibody. On the 30th day most of the animals were retested and the same studies made.

Text-fig. 1 summarizes the data obtained from this experiment. There is a rather prompt decrease in fever induced, occurring before appearance of detectable precipitins, and becoming minimal at the 7th to 10th day. Thereafter a gradual increase in the febrile response occurs so that on the 30th day resistance to the febrile response has almost completely disappeared. On the 1st day no animal receiving toxin showed leucocyte migration at the 5th hour. On the 2nd day, 2 of 4 animals showed distinct migration, while on the 3rd day all 4 showed migration. By the 4th day the degree of migration was comparable to that of normal leucocytes and continued at that level until repetitive injections of toxin were discontinued. Gradual loss of leucocyte resistance occurred over the ensuing 10 days until no migration was demonstrable 5 hours after toxin injection. The extent of migration is expressed in millimeters, but there was often slight variation among replicate slides so that the values have no absolute meaning. Precipitins did not appear until the 4th day (Table I). The precipitin titer is illustrated in Text-fig. 1 by arbitrary units representing the antigen dilution giving maximum precipitation with undiluted antiserum. This shows that the largest amount of precipitin appeared on about the 5th day and remained at approximately this level there-

after, though there was a slight decline toward the end of the 30 day period. This conforms with the findings of others which have been interpreted as showing no relationship between the level of antibody and resistance to bac-



TEXT-FIG. 1. Thirteen rabbits were given 25.0 μg. *Shigella* toxic antigen every day for 10 days. Individual animals were tested at intervals thereafter, and most retested at 30 days with the same dose of toxin. The effect of this treatment on leucocyte migration, febrile response, and precipitin titer is shown. The precipitin titer is given in arbitrary units of 1 to 4 plus, depending on the antigen dilution giving maximum precipitation with undiluted antiserum. ● indicates the fever index on individual animals, and the solid line depicts the general trend of fever indices during the experiment. ○ indicates the precipitin titer on individual rabbits, and the broken line depicts the general trend of precipitin titer during the experiment.

terial pyrogens (5–8, 15). Likewise, there appears to be no relation between resistance of leucocytes and precipitin titer.

A considerable degree of leucopenia was observed 1 hour after injection of the toxin. On day 1 this amounted to a 50 per cent fall of total count from the control leucocyte level. The fall varied from 30 to 60 per cent during the 10 days of repetitive injections and on day 10, at the time of maximum re-

sistance, there was still an average fall of 50 per cent. These results would indicate that although leucocytes show good migration from the leucocyte-platelet layer in resistant animals this is not accompanied by a lessening of the degree of leucopenia induced by toxin.

Figs. 1 to 3 are photomicrographs illustrating leucocyte migration in "slide cells" made from bloods of a normal rabbit, of an animal injected for the first time with toxin, and of a resistant animal. Much of the background in these photographs consists of fibrin network.

This experiment, which has been repeated twice with identical results, demonstrates that leucocytes of animals given daily injections of the toxic antigen develop resistance to the inhibitory effect of endotoxin on migration of centrifuged cells. Centrifuged resistant leucocytes migrate normally 5 hours after

TABLE I

Precipitin Data of Representative Animals Given 25.0 μg. of Shigella Toxic Antigen Every Day for 10 Days

Day	10*	5	2.5	1.2	0.6	0.3	0.15	0.08	0.04	0.02	0.01	0.005	0.0025	0.0012	0.0006
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	+	+±	++	++	+±	+	+	+	±	±	—	—	—	—	—
5	+++	++++	+++±	++	++	++	++	+	+	+	±	—	—	—	—
8	+++	+++±	++++	+++	+++	+++	++±	++±	++	+	+	±	—	—	—
9	+++±	+++±	++++	+++	+++	+++	+++	++	++	++	+	±	—	—	—
10	+++±	+++±	++++	+++±	+++	+++	+++	++	++	++	+	±	—	—	—
31	+	++	+++	+++	+++	++	+	±	—	—	—	—	—	—	—

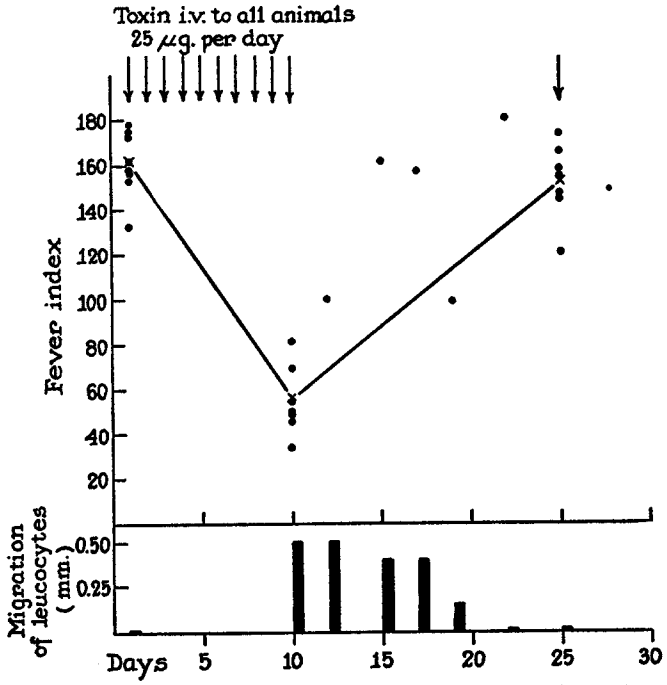
* Antigen expressed in milligrams per milliter. Tested with undiluted serum.

toxin administration. Resistance of leucocytes appears gradually and disappears 10 days after cessation of repetitive injections of toxin. There is a similarity in time of appearance and disappearance between resistance to the pyrogenic effect of endotoxin and resistance of leucocytes, but there is no apparent relationship of either to precipitin titer.

Experiment 2.—The effect of a second endotoxin was tested under similar conditions, though precipitin data were not obtained. 7 rabbits were given 25.0 μg. of P-35 intravenously every day for 10 days. All rabbits were tested for febrile response and leucocyte migration on day 1, 10, and 25. 5 animals were selected and tested individually on day 12, 15, 17, 19, and 22. In every instance leucocyte migration was compared with that of normal blood drawn at the same time. As in the experiment described above, leucocyte migration was studied 5 hours after injection of toxin. Leucocyte counts were done on the blood used to study migration.

The findings are summarized in Text-fig. 2. The febrile response is considerably lessened after 10 days of repetitive injection of P-35 toxin. The subsequent disappearance of this resistance seems more rapid than was ob-

served in animals made resistant to *Shigella* toxic antigen. All animals failed to show leucocyte migration when tested on day 1, whereas on day 10 all showed migration comparable to that of normal blood. The leucocyte resistance gradually disappeared over the ensuing 10 days. When all animals were retested on the 25th day none showed migration. Leucopenia was pronounced 1 hour after toxin injection, but the blood used for studying migra-



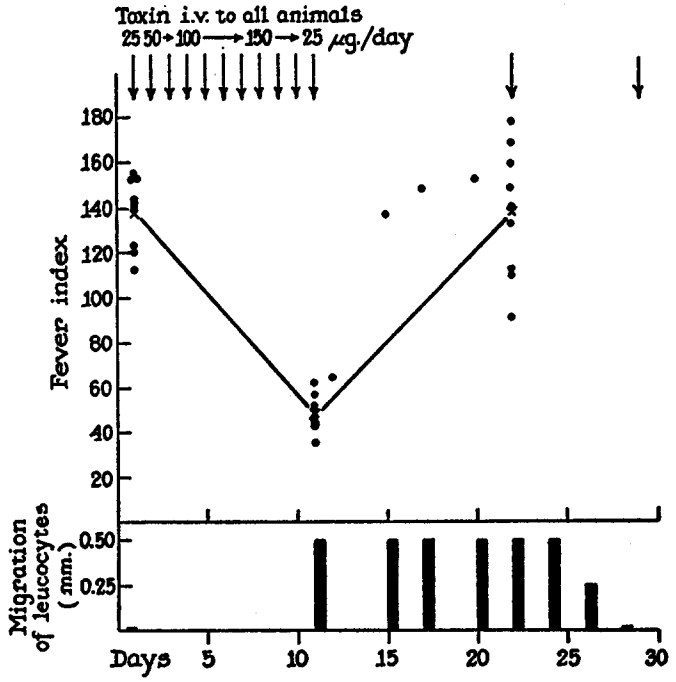
TEXT-FIG. 2. Seven rabbits were given 25.0 μg. of P-35 every day for 10 days. Individual animals were tested at intervals thereafter, and all retested at 25 days with the same dose of toxin. The effect of this treatment on leucocyte migration and fever response as indicated by the fever indices is shown. The solid line depicts the general trend of febrile response during the experiment.

tion, drawn at 5 hours, showed counts always within normal range or slightly elevated, usually with a preponderance of polymorphonuclear cells. In this experiment resistance to fever seemed to disappear more promptly than did leucocyte resistance.

The findings described correspond with those noted in the preceding experiment. Centrifuged leucocytes from animals resistant to P-35 had normal migration when tested after 10 days of repetitive toxin injection. Leucocyte resistance gradually disappeared during the 10 days following cessation of daily administration of toxin. The return of fever reactivity in these animals

was more prompt than in animals resistant to the *Shigella* toxic antigen, noted above. Repetition of this experiment has produced the same results.

Beeson (15) has found that rabbits given increasing doses of bacterial pyrogen every day for 10 to 14 days will show no fever at all when tested with the initial small dose of toxin. An attempt was made to repeat this obser-



TEXT-FIG. 3. Nine rabbits were given increasing doses of P-35 for 10 days. On day 11 all were given 25.0 μg. P-35. Individual rabbits were tested at intervals thereafter, all animals being retested on day 22. The effect of this treatment on leucocyte migration and febrile response is shown. The solid line depicts the general trend of febrile response, as indicated by fever indices, during the experiment.

vation in order to determine the effect of this complete resistance on leucocyte migration.

Experiment 3.—Nine rabbits were given P-35 polysaccharide every day for 11 days. On the 1st and 11th day they were given 25.0 μg., on the 2nd and 3rd day, 50.0 μg., on the 4th, 5th, 6th, and 7th day, 100.0 μg., and on the 8th, 9th, and 10th day, 150.0 μg. 4 rabbits were retested on day 12, 15, 17, and 19 with 25.0 μg. On the 22nd day all rabbits were tested again with 25.0 μg. P-35 intravenously. Temperatures, leucocyte migration, and white blood cell counts were studied as in the previous experiment. On the 24th, 26th, and 29th day 3 rabbits were tested individually for leucocyte migration.

Text-fig. 3 illustrates the data obtained from this experiment. When these animals were retested with a small dose of P-35, they were found not to have

the anticipated complete resistance to fever. Instead the degree of resistance and its disappearance closely resembled those of the preceding experiment. On the 1st day none of the rabbits showed leucocyte migration while on the 11th day all did, but to no greater or lesser degree than normal. This leucocyte resistance disappeared somewhat more slowly than in the preceding experiment, but because of the variability of the response of individual animals it cannot be concluded that this difference is significant.

Leucocyte Migration 1 Hour after Toxin Injection in Resistant Animals.—It has been suggested that resistance to the pyrogenic effect of the endotoxin may be related to more rapid removal of the toxic substance from the blood as a result of the conditioning effect of repeated exposure (16). If this were the case, it might be expected that leucocyte resistance would be less evident during the 1st hour after injection of the toxin. To test this possibility, studies were undertaken to determine if resistance of leucocytes was present at a time when the temperature of the resistant animal was maximal, usually 1 hour after injection of toxin.

Experiment 4.—Two rabbits were given 25.0 μg. of *Shigella* toxic antigen intravenously evrey day for 5 days and 2 rabbits every day for 6 days. On the 5th and 6th days, respectively, leucocyte migration was studied 1 hour after injection of the toxin. Temperatures were taken in the usual manner for 6 hours. Leucocyte counts were done on the blood used for study of migration. A normal animal was studied simultaneously for migration of leucocytes.

The 4 animals had temperatures of 40.7, 40.2, 41.2, and 40.8°C. at the time blood was drawn. This represented a rise in temperature of 1–1.5°C. for each animal. Leucocyte counts at that hour averaged 6000 per ml. for the 4 animals, no animal having a count below 5000 per ml. In all resistant animals migration equalled that of the normal animal studied simultaneously and that of blood drawn 5 hours after injection of toxin. The fever indices of these 4 rabbits were 39, 30, 19, and 21, whereas the indices on the 1st day of toxin administration were 167, 160, 129, and 100 respectively, indicating a considerable degree of fever resistance as well.

It is apparent, therefore, that blood from rabbits given injections of *Shigella* toxic antigen daily for 5 to 6 days shows migration of centrifuged leucocytes 1 hour after toxin injection comparable to that of untreated normal rabbits. This is at a time when fever is maximum in the resistant animal. It should be pointed out that centrifuged leucocytes from animals which have received toxin for the first time have never shown migration at 1 hour.

Passive Transfer of Pooled Serum from Resistant Animals.—Others have been unable to passively transfer resistance to bacterial pyrogens (6, 15). In addition, in the case of the Shwartzman phenomenon induced with endotoxin protection is not demonstrable by passive transfer of immune serum (5, 8). On the other hand, there is evidence that immune serum can serve to

passively transfer protection against the lethal effect of such toxin (9–12). In the following experiment the effect of passive transfer of serum from resistant animals was studied with respect to the effect on leucocyte migration.

TABLE II

Precipitin Data of Pooled Donor Serum and Recipient Serum to Shigella Toxic Antigen

Rabbit	10*	5	2.5	1.2	0.6	0.3	0.15	0.08	0.04	0.02	0.01	0.005	0.0025	0.0012	0.0006
Pooled donor serum	++	++++±	++++	++++±	+++	+++±	++	++	+	±	−	−	−	−	−
‡ 9-10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
‡ 9-11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
§ 9-10	−	++	+++	+++±	++	+	±	±	−	−	−	−	−	−	−
§ 9-11	−	++	+	±	−	−	−	−	−	−	−	−	−	−	−

* Shigella toxic antigen represented as milligrams per milliliter.
‡ Recipient animals' serum immediately before injection of donor serum.
§ Recipient animals' serum 1 hour after injection of donor serum.

Experiment 5.—Two rabbits were made resistant to *Shigella* toxic antigen by administration of 25.0 μg. every day for 14 days. The day following the last injection both rabbits were bled to death from the heart using precautions for asepsis. This blood was allowed to clot overnight in sterile vaseline-lined tubes. The serum thus obtained was pooled and used the following day for passive transfer to normal rabbits. Concomitantly 2 normal rabbits were bled to death and their serum pooled. 2 normal rabbits were given 25.0 ml. of the immune serum intravenously after removal of 10.0 ml. of blood from ear veins for control precipitin analysis. 2 additional normal rabbits were bled in a similar manner but given 25.0 ml. of normal serum. 1 hour later about 5.0 ml. of blood was collected from the ear veins of all 4 rabbits and then each was given 25.0 μg. *Shigella* toxic antigen intravenously. Temperatures were taken as previously described and leucocyte migration was studied in the animals 5 hours after the toxin was given. A normal rabbit was bled at the same time for comparison of leucocyte migration.

Table II presents the precipitin data of the pooled serum from resistant donor animals and the data obtained from the serum of the recipient animals before and 1 hour after the passive transfer of serum. There was transfer of appreciable amounts of precipitin. Leucocyte migration was absent in all 4 animals after receiving toxin. The fever indices of the 2 animals receiving immune serum were 110 and 112. The fever indices of the 2 animals receiving normal serum were 100 and 123, indicating there had been no transfer of resistance to febrile response. Thus, it was not possible to transfer leucocyte or fever resistance in this study.

Abolition of Resistance by Thorotrast.—Resistance to febrile response and the Shwartzman reaction due to endotoxins can be abolished by the intravenous injection of thorotrast (8, 14, 16, 20). It has been suggested that this action of thorotrast is by blockade of the reticulo-endothelial system. Trypan blue will accomplish the same effect but to a smaller degree. The hypothesis

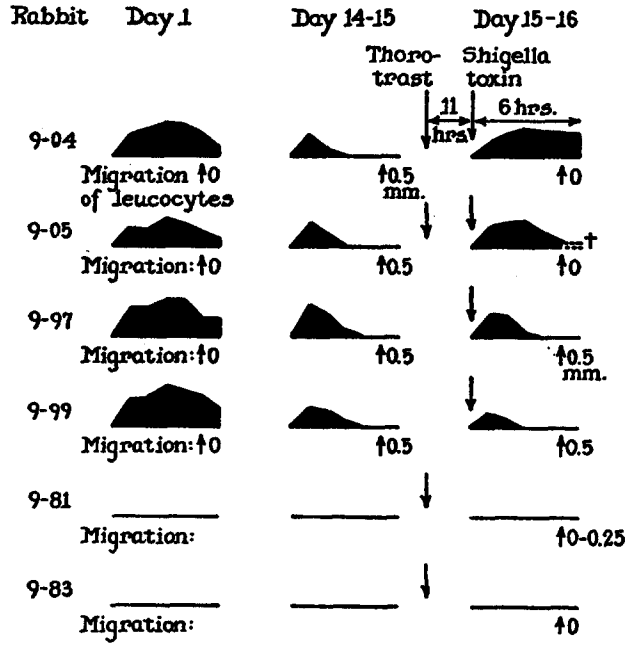
has been suggested that resistance may be dependent on removal of the endotoxin by cells of the reticulo-endothelial system and that blocking agents act by preventing the functioning of these cells.

Experiment 6.—Four rabbits were made resistant to *Shigella* toxic antigen by the administration of 25.0 μg. every day for 14 to 15 days. 12 hours after the last dose of toxin, 2 of the 4 rabbits were given 9.0 ml. of thorotrast intravenously. At the same time 2 normal rabbits were given 9.0 ml. of thorotrast intravenously. 11 hours after administration of thorotrast the 4 resistant animals were given 25.0 μg. of *Shigella* toxic antigen intravenously. Temperatures were recorded as previously. Blood was drawn from all 6 rabbits by cardiac puncture 5 hours after the toxin had been injected for studies of leucocyte migration. A normal untreated rabbit was bled at the same time. White blood cell and differential counts were done on the cardiac blood of each animal. 3 rabbits which received thorotrast died following completion of the experiment. These were autopsied, tissues fixed in 10 per cent formalin, and appropriate sections stained with hematoxylin-eosin. Crystals of hematin were removed from the sections prior to staining (21).

Of the 4 rabbits rendered resistant to *Shigella* toxic antigen, all showed no migration when tested on day 1 (Text-fig. 4). By the 14th and 15th day, they showed migration comparable to that of normal rabbits. There was again considerable decline in febrile response during this period as well. After the administration of thorotrast to the 2 resistant animals, resistance to both the fever-inducing action and the leucocyte effect of toxin was abolished. The 2 resistant animals receiving toxin alone continued to show minimal febrile response and the leucocytes migrated normally. Although thorotrast appeared to abolish resistance to the effect of toxin on leucocyte migration, this agent when injected alone impaired the migration of leucocytes from the centrifuged leucocyte-platelet layer. This finding was confirmed in 2 additional animals studied. However, these leucocytes showed normal ameboid activity when observed on a warm stage prior to centrifugation or when resuspended by shaking after centrifugation, and in this respect the action of thorotrast resembles the effect of endotoxins on leucocytes (4). The exact significance of this finding remains to be elucidated. Leucocyte counts of cardiac blood were elevated or within normal limits. Most often there was neutrophilia as well. Histopathological studies of animals dying spontaneously revealed massive accumulation of thorotrast in the spleen and liver. There were accumulations of thorotrast in the phagocytes of the adrenal gland. In the lung there were numerous macrophages containing thorotrast, particularly around the blood vessels. Of interest was the presence of thorotrast in leucocytes in the blood vessels of all organs, particularly lung and liver. In the kidney small foci of hemorrhage were rarely seen and most glomeruli contained cells filled with thorotrast. The blood vessels of the lung were often dilated and contained aggregations of leucocytes. The blood vessels of the liver were marekdly dilated and around the portal triads there were often hemorrhage and marked accumulation of phagocytes containing thorotrast. Near the portal triads foci

of hemorrhage and necrosis were seen in the liver lobules, surrounded by polymorphonuclear leucocytes. The hemorrhagic and vascular changes were seen only in those animals receiving toxin as well as thorotrast.

In view of the fact that thorotrast impairs the migratory capacity of centrifuged leucocytes, this experiment does not provide clear evidence that this substance abolishes the resistance of leucocytes to endotoxin. The expected

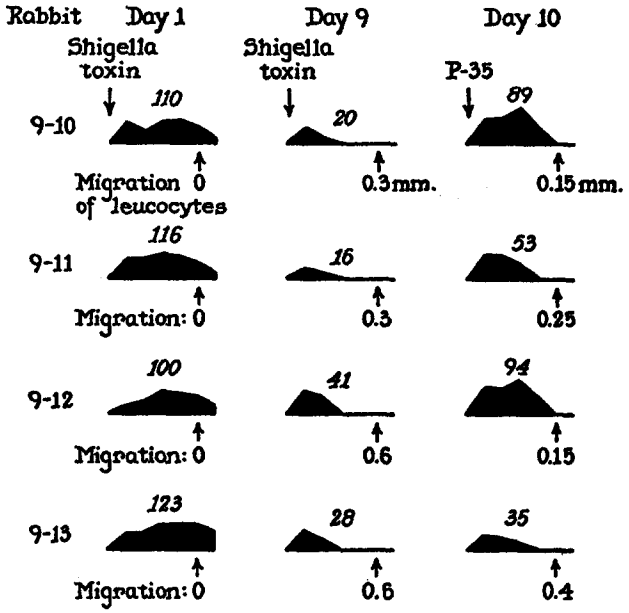


TEXT-FIG. 4. Febrile responses are indicated by the solid blocked areas representing plotted fever curves. Four rabbits were given 25.0 μg. *Shigella* toxic antigen intravenously every day for 14 to 15 days. 2 of these and 2 normal rabbits were then given 9.0 ml. of thorotrast intravenously. 11 hours later the 4 resistant rabbits were given 25.0 μg. *Shigella* toxin. The effect of thorotrast on resistance of leucocytes and pyrogen tolerance, and the effect of thorotrast alone on leucocyte migration are shown.

loss of resistance to the pyrogenic effect of endotoxin occurred after injection of thorotrast.

Non-Specificity of Resistance to Bacterial Endotoxins.—Other studies have shown that animals rendered refractory to the Shwartzman reaction by repeated injection of an endotoxin are likewise resistant to heterologous endotoxins (8). Similarly, rabbits made resistant to the pyrogenic properties of one endotoxin become somewhat resistant to others (6, 7, 15, 17). The non-specificity of the leucocyte resistance was studied in the following experiments.

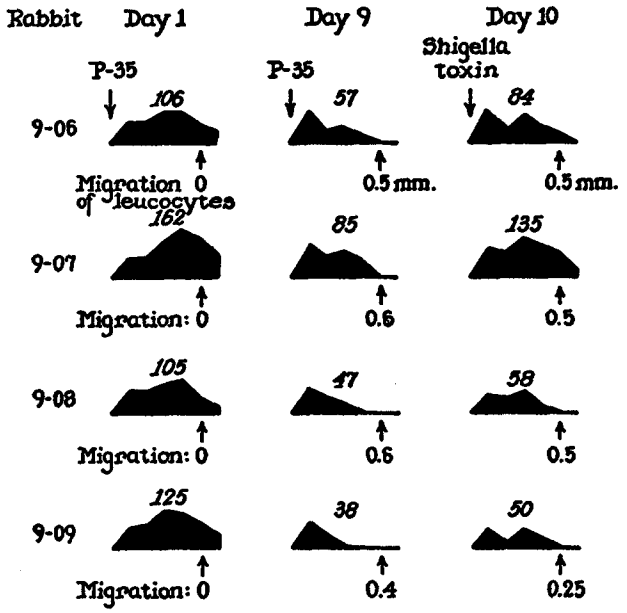
Experiment 7.—Four rabbits were given 25.0 μg. of *Shigella* toxin every day for 9 days. On the 10th day all were challenged with 25.0 μg. P-35 intravenously. Temperatures were taken and recorded in the manner already described. Leucocyte migration was studied 5 hours after injection of toxin on day 1, 9, and 10. Leucocyte counts were done on the cardiac blood used for studying migration.



TEXT-FIG. 5. Fever responses are indicated by the solid blocked areas representing plotted fever curves, and the corresponding fever indices. Four rabbits were given 25.0 μg. *Shigella* toxic antigen every day for 9 days. On day 10 these animals were given 25.0 μg. P-35. The cross-resistance to the 2 toxins is illustrated by febrile response and leucocyte migration.

There was a pronounced decline in febrile response in the animals on day 9 and leucocyte migration was normal (Text-fig. 5). When challenged on the next day with a heterologous endotoxin, P-35, there was an increase in the degree of febrile response but the temperatures in all animals had returned to normal before the 5th hour. Fever indices indicate the presence of some but not complete cross-resistance. With the pyrogen cross-resistance, there is likewise some leucocyte cross-resistance, though not complete, to the heterologous endotoxin. In this experiment all resistant animals tested with a different endotoxin showed leucocyte migration whereas normal animals tested with this toxin for the first time have never shown migration from the leucocyte-platelet layer at 5 hours. However, the leucocyte migration with the heterologous toxin was not equal to that of a normal animal. Leucocyte counts at the 5 hour period were normal or elevated, with an equal number or preponderance of polymorphonuclear cells.

Experiment 8.—Four rabbits were made resistant to P-35 by daily intravenous injection of 25.0 μg. for 9 days. On day 10 all were challenged with 25.0 μg. of *Shigella* toxin. Temperatures were taken and recorded as before. Leucocyte migration was studied 5 hours after injection of toxin on days 1, 9, and 10, and compared with that of a normal control. Temperatures were recorded on these same days. Leucocyte counts were done on cardiac blood used for studying migration.



TEXT-FIG. 6. Fever responses are indicated by the solid blocked areas representing plotted fever curves, and the corresponding fever indices. Four rabbits were given 25.0 μg. of P-35 every day for 9 days. On day 10 these animals were given 25.0 μg. *Shigella* toxic antigen. The cross-resistance to the 2 toxins is illustrated by febrile response and leucocyte migration.

The findings are seen to be essentially the same as in the preceding experiment (Text-fig. 6). There is some cross-resistance, though not complete, to fever and to the effect on leucocytes. Leucocyte counts were normal or slightly elevated in these animals 5 hours after toxin injection.

DISCUSSION

It was shown in the preceding paper that the injection of bacterial endotoxin into a rabbit results in the inhibition of migration of leucocytes from centrifuged blood, and the possible significance of this effect was discussed in relation to the Shwartzman reaction (4). The present paper deals with the inhibition of the leucocyte response by repeated injection of endotoxin.

Previous work has established that repetitive injection of endotoxin into a

rabbit makes the animal resistant to the Shwartzman reaction and to the febrile response induced by the toxin (6–8, 14–17). This resistance seems to be non-specific, in that resistance to one endotoxin confers resistance to others, and appears to be unrelated to antibody titer. Transient leucopenia induced by the injection of endotoxin is not affected by repeated injection of toxin, and develops to a similar degree in the resistant animal. The nature of the resistance to endotoxin is inadequately explained but has been thought to be related to a conditioning of the reticulo-endothelial system (16).

The studies reported here demonstrate that coincident with the development of tolerance to the febrile response caused by endotoxin, migration of leucocytes from centrifuged rabbit blood is no longer inhibited by injection of the toxin. The mechanism of the failure of endotoxin to affect leucocytes of resistant animals is not clear. A possible explanation is the neutralization of the toxin by serum of the resistant animal, but this is not supported by the failure to transfer resistance passively with large amounts of antiserum. A second possible mechanism is more rapid removal of the toxin from the blood by the reticulo-endothelial system. Blockade of the reticulo-endothelial system in an attempt to evaluate this possibility was not satisfactory since the thorotrast used for this purpose proved to have an effect on leucocytes similar to that of endotoxin. A third conceivable explanation for the failure of toxin to inhibit migration of leucocytes from centrifuged blood of the resistant animal is that the leucocytes themselves become adapted to the endotoxin by repetitive exposure. The gradual disappearance of resistance could then be explained by the replacement of resistant cells by newly formed cells not adapted to the toxin. In the preceding paper (4) it was shown that plasma of a non-resistant animal given endotoxin intravenously did not contain material capable of interfering with the migratory activity of normal cells, and it would thus seem unlikely that repetitive injection of toxin could have exhausted any plasma factor which in combination with the toxin could interfere with leucocyte activity. At present, it seems most reasonable to assume that the failure of toxin to inhibit migration of leucocytes from resistant rabbits is due either to (*a*) the presence of resistant leucocytes which have become adapted to the toxin by repetitive exposure, or (*b*) rapid removal of the toxin by the reticulo-endothelial system, thereby protecting the leucocyte from injury by the toxin. The failure to demonstrate toxic inhibition of leucocyte migration a short interval after injection into the resistant animal does not disprove the latter hypothesis.

Why resistant animals continue to show a degree of leucopenia comparable to that of non-resistant animals when injected with endotoxin is obscure. The presence of leucocytes which are resistant to the effect of endotoxin on their migration does not dismiss the possibility of their being injured in another way. Sequestration of leucocytes in the lung vascular bed still could be related

to removal of damaged cells, or to an alteration of the function of the blood vessels themselves.

The studies reported here confirm the previous findings (14, 15) of abolition of resistance to the endotoxin-induced fever by thorotrast, and of the non-specificity of the resistance to endotoxins of heterologous species of organisms. This non-specificity is shown to apply also to the inhibition of the effect of endotoxins on leucocyte migration. The fact that the titer of antibody to the endotoxin is not related to the degree of resistance to its fever-inducing activity is confirmed, and the same lack of correlation is found between antibodv titer and leucocyte resistance.

The leucocyte has been implicated in the pathogenesis of the Shwartzman phenomenon (2, 3), and the presence of leucocytes resistant to endotoxin may be responsible in part for the development of resistance to the Shwartzman reaction. However, it is unlikely that the presence of resistant leucocytes is in any way responsible for tolerance to the fever-inducing activity of toxin, since a leucopenic animal given endotoxin develops fever comparable to the normal rabbit given toxin.

SUMMARY

Bacterial endotoxin injected intravenously into rabbits inhibited the migration of leucocytes from the buffy coat of centrifuged blood (4). Repeated daily injections of endotoxin resulted in the rabbits becoming resistant to the fever-inducing action of the toxin, and migration of leucocytes from centrifuged blood was no longer inhibited by injection of the toxin. Leucocyte migration from the buffy coat of centrifuged blood after injection of toxin into the rabbits appeared gradually over the first few days of repeated injections, and disappeared during the 10 to 15 days after cessation of daily injections of toxin. The resistance to endotoxin, demonstrated by leucocyte migration and pyrogen tolerance, could not be passively transferred with serum from resistant animals, and was non-specific, in that resistance to one endotoxin conferred some resistance to toxin from an organism of a different species. No relationship could be demonstrated between precipitin titer and resistance. Thorotrast abolished resistance to the fever-inducing activity of endotoxin, but its effect on leucocyte resistance was not clear, since when injected alone it inhibited migration of leucocytes from the buffy coat of centrifuged blood.

The suggestion is made that the failure of toxin to inhibit the migration of leucocytes from resistant rabbits is due either to the presence of leucocytes which have become adapted to the toxin by repeated exposure, or to rapid removal of the toxin by the reticulo-endothelial system. It is unlikely that leucocyte resistance participates in the development of tolerance to the fever-inducing action of endotoxin. However, in view of the participation of the leucocyte in the pathogenesis of the Shwartzman reaction, the presence of

leucocytes resistant to endotoxin may be responsible in part for the development of resistance to the Shwartzman phenomenon.

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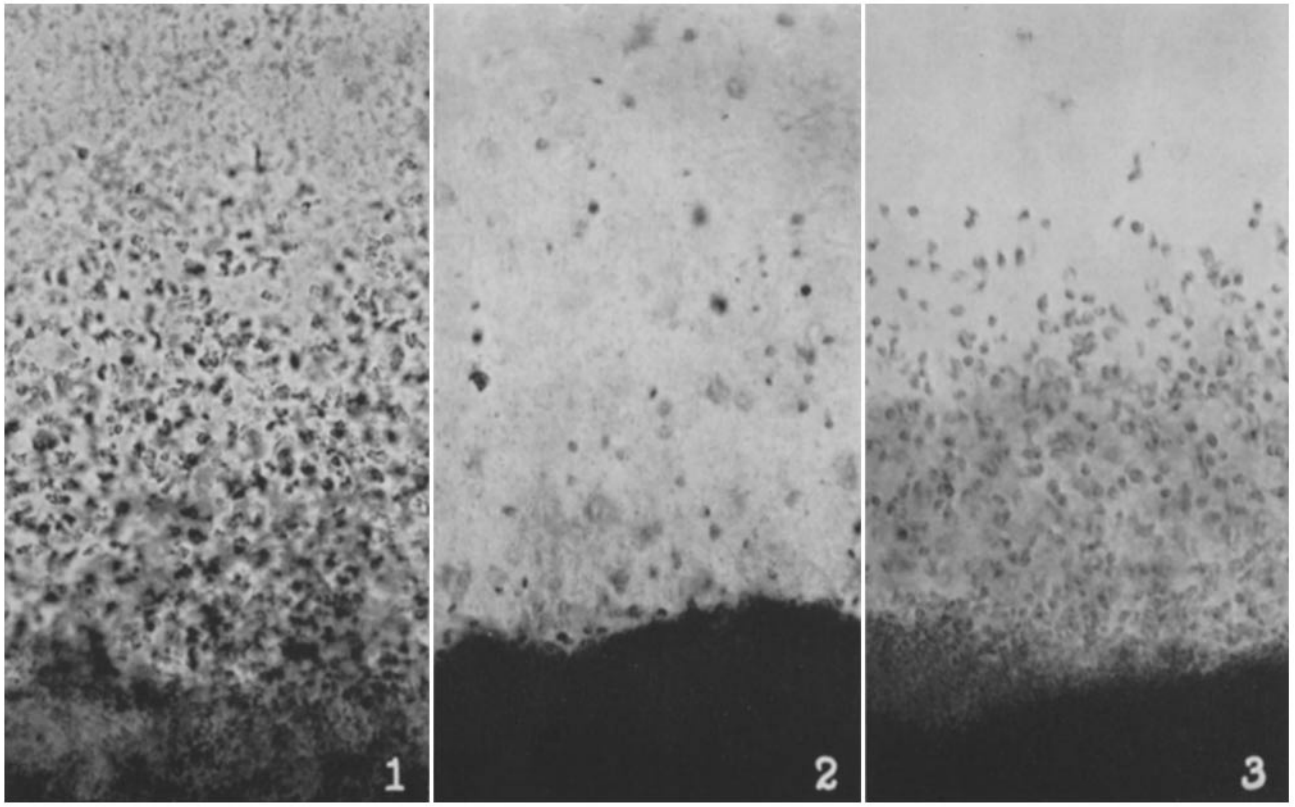
EXPLANATION OF PLATE 44

The photographs were made by Mr. Julian A. Carlile.

FIG. 1. Migration of normal leucocytes from buffy coat after 1 hour incubation. × 175.

FIG. 2. Blood drawn from rabbit 5 hours after injection of 25.0 μg. *Shigella* toxic antigen. Absence of migration of leucocytes from buffy coat after 1 hour incubation. × 175.

FIG. 3. Blood drawn from rabbit resistant to *Shigella* toxic antigen 5 hours after injection of 25.0 μg. of the toxin. Migration of leucocytes from buffy coat after 1 hour incubation. × 175.



(Cluff: Effect of endotoxins on leucocytes. II)