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STUDIES ON HUMAN LEUKOCYTE MOTILITY. II. EFFECTS OF BACTERIAL ENDOTOXIN ON LEUKOCYTE MIGRATION, ADHESIVENESS, AND AGGREGATION‡

While polymorphonuclear leukocytes appear to play a major role in many of the biologic effects of bacterial endotoxin,[1,2] the mechanism by which endotoxin interacts with these cells is not clear. Recent studies from this laboratory showed that phagocytosis of several different types of particles inhibited neutrophil migration in microhematocrit tubes.[3] This inhibition was due to both decreased motility of individual neutrophils participating in phagocytosis and aggregation of leukocytes into clumps. It was subsequently observed that exposure of neutrophils to bacterial endotoxin or immune precipitates produced similar effects.

The present studies were undertaken to test the thesis that the changes observed in neutrophil function after incubation with endotoxin result from phagocytosis of endotoxin and represent a general pattern of polymorphonuclear leukocyte response to ingestion of a variety of macromolecular substances.

MATERIALS AND METHODS

The basic system used for study of neutrophil migration was initially employed by Ketchel and Favour,[4] and has been previously described.[3] Blood from normal donors was anticoagulated with heparin in a final concentration of 40 μg. per ml. Drummond 32 mm. microhematocrit tubes were two-thirds filled with blood, the bottom end flame-sealed, and centrifuged for two minutes in a Drummond microhematocrit centrifuge. The tubes were then placed in a vertical position and the distance traversed by the advancing cells over a four-hour period was measured microscopically, using an ocular micrometer. Nine or ten microhematocrit tubes were employed for each variable tested. Individual cell motility was assessed by oculometer measurement (970x magnification) of the total linear motion of individual non-aggregated leukocytes over a 5-minute period. Temperature was maintained at 37° ± 0.5°C.

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Leukocyte aggregation was assessed by a minor modification of this technique. Specimens were similarly prepared and centrifuged but then inverted for one hour to redistribute the buffy coat throughout the plasma. Tubes were then laid horizontally in the bottom of large centrifuge cups and centrifuged at 1,800 *g* for two minutes. This permitted adherence of leukocytes to the sides of the capillary tubes. Erythrocytes were removed from the area under observation by a 10-second vertical centrifugation in the Drummond centrifuge. A drop of oil placed over the capillary tubes eliminated optical distortion and allowed direct microscopic observation of leukocytes adhering to the sides of the microhematocrit tubes. The percentage of leukocytes in clumps was counted as an index of leukocyte aggregation.

The effects of endotoxin on over-all cell migration, leukocyte aggregation, and individual leukocyte motility were determined in the following manner: Blood was mixed with endotoxin, placed in sterile stoppered siliconized tubes, and rotated in a 37°C. incubator for 30 minutes. Aliquots of blood, to which saline rather than endotoxin had been added, were treated in identical fashion and served as controls. Following this incubation, the control and experimental samples were placed in microhematocrit tubes and studied as described above.

An *E. coli* endotoxin of the Boivin type (Difco *E. coli* 0127:B8) suspended in isotonic saline in a final concentration of 100 μg per ml. was used. Individual modifications of the endotoxin incubation period are described under results.

The effect of heat-labile plasma factors on leukocyte-endotoxin interactions was determined by studying migration of neutrophils in endotoxin-free plasma after preincubation with endotoxin in heated plasma. Two milliliters of fresh heparinized blood were mixed with 5 ml. of saline containing 100 mg/100 ml. glucose and 20 per cent of either heated (56°C. for 30 minutes with centrifugation to remove insoluble precipitate) or fresh plasma. These mixtures were then centrifuged for two minutes at 3,000 r.p.m., the plasma-saline supernate aspirated, and 1 ml. of either heated or fresh plasma added to restore a normal hematocrit. This resulted in two similar aliquots of blood, except that in one the plasma used both in washing and resuspension had been heated. Specimens were mixed with one part endotoxin suspension per nine parts blood and then rotated at 37°C. Immediately before and 2½, 5, and 10 minutes after adding endotoxin and beginning rotation, aliquots were removed from each of the flasks. The plasma was removed from these aliquots by centrifugation, the cells washed once in saline containing 30 per cent normal plasma, the washed cells resuspended in fresh plasma restoring a normal hematocrit, and leukocyte migration then determined.

The effect of antigen-antibody interaction on leukocyte function was determined by incubating normal blood with egg albumin in a final concentration of 25 μg per ml. and egg albumin-immune rabbit plasma in a final concentration of 2.5 per cent. Controls consisted of similar aliquots of blood containing only saline, saline plus immune rabbit plasma, and egg albumin plus non-immune rabbit plasma. Preliminary experiments demonstrated that the concentration of rabbit plasma was critical, larger amounts causing spontaneous aggregation of neutrophils. All samples were rotated at 37°C. for ten minutes and then employed in migration and motility studies. Leukocyte aggregation in the sample containing antigen and antibody was so extensive that accurate measurement could not be made in the capillary tubes. Accordingly, this measurement was made on coverslip preparations of the incubated samples. Statistical significance of results was determined by t-test analysis.

Hyper-immune plasma was obtained from rabbits injected with at least three weekly subcutaneous injections of egg albumin in Freund's complete adjuvant (ovalbumin, recrystallized x 2. Worthington Biochemicals, Freehold, New Jersey). Each injection contained 25 mg. of egg albumin in a 2 ml. volume.

RESULTS

Table 1 illustrates that neutrophils in blood preincubated with endotoxin become adherent to one another and that individual neutrophil motility is

TABLE 1. MODIFICATION OF NEUTROPHIL FUNCTION BY ROTATION WITH ENDOTOXIN

Endotoxin	*Rotation (37°-30 min)*	*Neutrophil migration (4-hour)*	*Neutrophil aggregation*	*Individual neutrophil motility*
(μg/ml. blood)		(mm.)	(per cent)	(oculometer units per 5 min.)
0	yes	1.87	0	131
1	no	1.47	0	133
1	yes	1.05	2	90
10	no	.75	23	56
10	yes	.09	57	—
100	no	.14	75	8
100	yes	.07	77	2.5

decreased. These effects occur rapidly (within 15 seconds), are produced by small concentrations of endotoxin, and are enhanced by rotation. Similar changes in leukocyte function have been observed after incubation of blood with live staphylococci, heat-killed staphylococci, or latex particles. Enhancement of cell injury by rotation with endotoxin may be attributed to increased opportunity for cell particle contact under conditions favoring phagocytosis. High concentrations of endotoxin (100 μg per ml. blood) produced maximal effects even in the absence of rotation during preincubation.

Preparation of capillary tubes for migration studies involves centrifugation which may increase cellular aggregation. The control studies, however, demonstrate that centrifugation does not of itself produce such aggregation. In order to establish that centrifugation was not necessary for leukocyte injury by endotoxin, individual neutrophil motility of noncentrifuged specimens was studied on coverslip preparations after rotation with a 10 μg/ml. concentration of endotoxin for 30 minutes at 37°C. Control specimens were treated identically and received saline additives. Control neutrophils traversed 46 ± 1 (mean ± S.E.) oculometer units per 2.5

minute period; rotation of neutrophils with endotoxin reduced this distance to 17 $\pm$ 1 (mean $\pm$ S.E.) oculometer units per 2.5 minute period ($p < .001$).

Effect of EDTA on the leukocyte-endotoxin interaction

Phagocytosis of bacteria by neutrophils is inhibited by sodium EDTA. It was reasoned that inhibition of the neutrophil-endotoxin interaction by sodium EDTA would provide further indirect evidence that this interaction is essentially a phagocytic event. Because high concentrations of sodium EDTA also completely eliminate adherence of neutrophils to glass, making estimation of neutrophil migration impossible, a two-phase experiment was designed. Blood was first incubated with endotoxin in the presence of sodium EDTA, the plasma then removed by centrifugation, the cells washed once in Hanks' solution containing 30 per cent normal autologous plasma and 100 mg/100 ml. glucose, and the hematocrit then restored with normal heparinized plasma. Table 2 demonstrates that

TABLE 2. EFFECT OF EDTA ON LEUKOCYTE-ENDOTOXIN INTERACTION

Additives during pre-incubation*		Studies after suspending pre-incubated cells in normal plasma					
		Neutrophil migration (4-hour)		*Neutrophil aggregation*		*Individual neutrophil motility*	
Endotoxin (μg per ml.)	*Sodium EDTA* (mM)	(mm)	(p**)	(per cent)	(p**)	(oculometer units per 5 min.)	(p**)
0	10	.49		0		60	
			=.1		.05		=.1
10	10	.45		6		70	
			<.001		<.001		<.001
10	0	.09		47		5	

* 0.1 ml. of the indicated additives mixed with 0.8 ml. heparinized blood. Final 1.0 ml. specimen rotated for 30 minutes at 37°C.

** Determined by t-test.

sodium EDTA in concentration sufficient to bind all divalent cation prevents endotoxin-induced neutrophil aggregation, impairment of migration, and decreased motility of individual non-aggregated neutrophils. Phagocytosis of bacteria and the neutrophil aggregation that is associated with bacterial phagocytosis are similarly inhibited by these concentrations of sodium EDTA.

Effect of temperature and of heat inactivation of plasma on cell injury by endotoxin

Phagocytosis of bacteria by leukocytes does not take place in the cold. Similarly, when blood is incubated with endotoxin at 4°C., the cells then washed as previously described, and migration studied after the addition of heparinized autologous plasma to restore a normal hematocrit, the effects of endotoxin are not seen (Table 3).

TABLE 3. MODIFICATION OF ENDOTOXIN EFFECTS BY TEMPERATURE

Additive during pre-incubation		Temperature of pre-incubation	Neutrophil migration (4 hour at 37°C. in endotoxin-free plasma)
Endotoxin (μg per ml.)	*Saline*	*Degrees centigrade*	(mm)
0	+	4°	1.18
10	—	4°	1.40*
0	+	37°	1.05
10	—	37°	0.02*

* Difference between these values significant at $<.001$ probability level.

Since phagocytosis is accelerated by heat-labile plasma factors, the effects of heat inactivated plasma on the endotoxin-neutrophil interaction were assessed. Inhibition of neutrophil migration by endotoxin was more prompt in fresh plasma than in heated plasma (Fig. 1). This difference was most apparent in specimens removed from rotation-incubation with endotoxin after 2.5 and 5 minutes. After 10 minutes of incubation, no significant difference between cells exposed to endotoxin in heated plasma and cells exposed to endotoxin in fresh plasma was seen. The dosage of endotoxin used was also critical in demonstrating this effect. When large amounts of endotoxin were used, migration was maximally inhibited in either heated or fresh plasma.

Granulocyte degranulation by endotoxin

Leukocytes were obtained from rabbit heparinized blood by dextran sedimentation and differential centrifugation,[5] washed twice in saline containing 30 per cent plasma, and resuspended in fresh plasma to a final concentration of 1 x 10^7 leukocytes per ml. Aliquots were mixed with a saline suspension of endotoxin (one part per 9 parts leukocyte-plasma) and with control amounts of saline, and rotated at 37° C. for 15 minutes. Wright-stained coverslip preparations were then examined and the re-

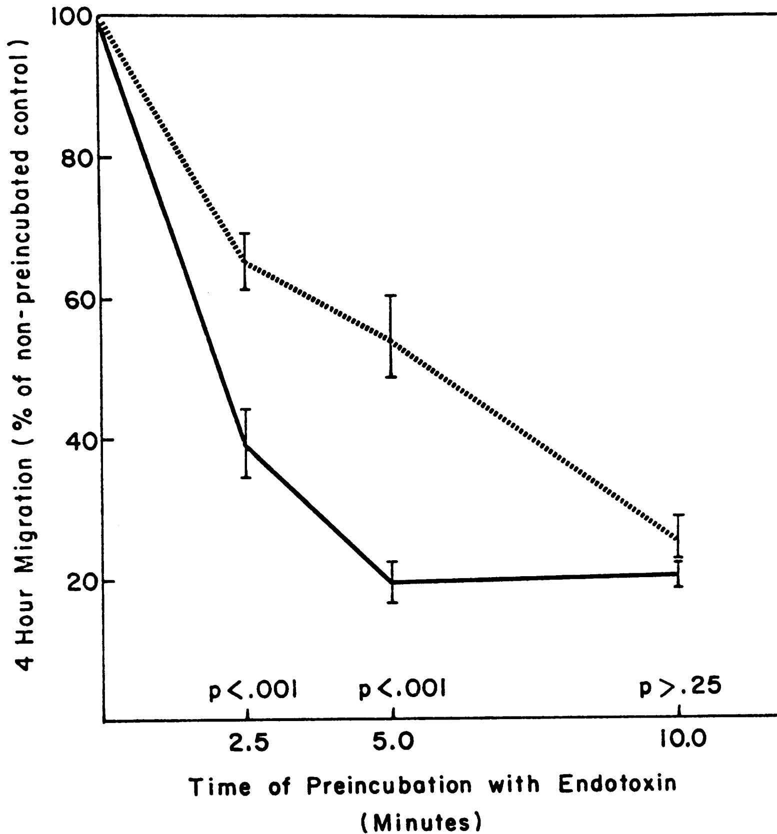

FIG. 1. Effect of heated plasma or fresh plasma on the neutrophil-endotoxin interaction. Migration of neutrophils preincubated with 5 μg per ml. of endotoxin in heated plasma (dotted line) is compared with migration of neutrophils preincubated with 5 μg per ml. of endotoxin in fresh plasma (solid line). Brackets represent standard error of the mean.

maining neutrophil granules were enumerated.[6] Table 4 demonstrates that significant degranulation was seen in the specimen containing endotoxin.

TABLE 4. EFFECT OF ENDOTOXIN ON DEGRANULATION OF RABBIT NEUTROPHILS

Endotoxin concentration	*Granules/neutrophil*
(μg per ml.)	
0	26 ± 3*
10	7 ± 2*

* Standard error of mean of 20 cells ($p < .001$).

Effect of centrifugation on endotoxin

Saline containing 100 μg/ml. *E. coli* 0127:B8 endotoxin was incubated for 15 minutes at 4° C. or centrifuged at 28,000 *g* for 15 minutes at 4°C. Then 0.2 ml. supernatant of these solutions was added to 2.0 ml. of blood and rotated 15 minutes at 37°C. The control specimen received 0.2 ml. of endotoxin-free saline. Leukocyte migration studies were performed and expressed as per cent of control (Table 5).

TABLE 5. EFFECT OF CENTRIFUGED ENDOTOXIN ON LEUKOCYTE MIGRATION

Additives during pre-incubation of blood and endotoxin	*Leukocyte migration (per cent of control)*
10 μg/ml. endotoxin*	16†
Supernatant of 10 μg/ml. endotoxin**	96†

* Saline containing 100 μg/ml. of endotoxin was incubated at 4°C. for 15 min.; 0.2 ml. of this mixture was then added to 2.0 ml. heparinized blood and rotated 15 min. at 37°C.

** Saline containing 100 μg/ml. of endotoxin was centrifuged at 28,000 *g* for 15 min. at 4°C. 0.2 ml. of supernatant was then added to 2.0 ml. heparinized blood and rotated as above.

† ($p < .001$) by t-test.

Migration was inhibited by non-sedimented endotoxin but not by the supernatant of the endotoxin that had been centrifuged.

Effect of immune precipitates on leukocyte function

When human leukocytes were exposed to egg albumin and small quantities of plasma from rabbits immunized with egg albumin, the changes in neutrophil migration, aggregation, and individual cell motility were similar to those produced by phagocytosis of bacteria, phagocytosis of latex particles, and incubation with endotoxin (Table 6). Marked inhibition of migration, leukocyte aggregation, and inhibition of individual cell motility was observed in samples containing both antigen and antibody. Inhibition of neutrophil migration was also observed when antigen and immune rabbit plasma were first incubated together for 10 minutes (37°C.) and then added to human blood. The immune precipitates that formed under these circumstances retained the capacity to inhibit neutrophil migration after centrifugation and resuspension in saline, whereas the supernate in which the precipitates were formed did not. (Table 7).

TABLE 6. EFFECT OF ANTIGEN-ANTIBODY INTERACTIONS ON NEUTROPHIL FUNCTION

*Additives during pre-incubation**				*Studies after pre-incubation*		
	Rabbit plasma					
Egg albumin	*Non-immune*	*Immune*	*Saline*	*Neutrophil migration*	*Neutrophil aggregation*	*Neutrophil motility*
(25 μg/ml.†)	(2.5%†)	(2.5%†)		(mm/ 4 hours)	(Per cent)	(oculometer units/5 min.)
0	0	0	++	2.2	2.7	104
+	0	0	+	2.1	6.7	108
0	+	0	+	2.0	3.3	111
0	0	+	+	1.9	6.9	111
+	+	0	0	2.2	5.6	117
+	0	+	0	0.3‡	62.7§	37‡

* 0.05 ml. additives were mixed with 2.0 ml. blood.
† Final concentrations in blood.
‡ $p < .001$ by t-test.
§ $p < .001$, chi square test.

TABLE 7. EFFECT OF IMMUNE PRECIPITATES ON NEUTROPHIL MIGRATION

Additives during preincubation of blood (rotated at 37°C. for 10 minutes)	*Leukocyte migration (mm/4 hrs ± SE*)*
Saline	1.14 ± .04
Egg albumin (25 μg/ml. blood)	1.13 ± .03
Immune plasma (2.5% final concentration)	0.94 ± .04
Egg albumin (25 μg/ml.) + immune plasma (2.5%)	0.08 ± .03**
Supernatant of egg albumin + immune plasma‡	0.93 ± .03
Sediment of egg albumin + immune plasma‡	0**

* Standard error of the mean.
** $p < .001$ by t-test.
‡ Egg albumin (25 μg per ml.) and immune rabbit plasma (2.5% in saline) were incubated at 37°C. for 10 minutes. The resulting precipitate was centrifuged at 28,000 *g* for 10 minutes and resuspended in 1 ml. saline. The supernate and the resuspended immune precipitate were then each incubated with blood, and leukocyte migrations studied.

DISCUSSION

The present studies have measured changes in neutrophil migration, neutrophil aggregation, and changes in motility of individual non-aggregated neutrophils as indices of the interaction between endotoxin and neutrophils. Changes observed in these parameters of neutrophil function appear identical to changes observed after incubation of blood with viable bacteria, non-viable bacteria, and latex particles.[8] Factors known to modify *in vitro* phagocytic systems such as rotation during incubation, low tem-

perature, and chelation of divalent cations modified the effects of endotoxin on neutrophils in a manner consistent with the thesis that the effects of endotoxin-neutrophil interaction are also based on phagocytosis. In similar fashion, pretreatment of plasma by heating retarded the speed of the neutrophil-endotoxin interaction, as it is known to retard phagocytosis of other particles.[7,8]

The observation that phagocytosis inhibits neutrophil migration is not new. In 1949, Allgower and Bloch reported that neutrophil migration was suppressed by phagocytosis of virulent tubercle bacilli and freshly isolated strains of *S. aureus*.[9] Studies by Martin, Pierce, Middlebrook, and Dubos, using virulent and avirulent tubercle bacilli and experimental techniques quite similar to the ones employed in the present experiments, confirmed these findings, noted that inhibition of migration was associated with neutrophil aggregation, and indicated that this aggregation involved cells free of bacilli as well as cells containing bacilli.[10] Subsequently, Martin and Chaudhuri made similar *in vitro* observations using bacterial endotoxins.[11]

The effect of bacterial endotoxin injections on migration of rabbit neutrophils was studied extensively by Berthrong and Cluff.[12] They concluded that endotoxin caused increased stickiness of cell surfaces rather than decreased intrinsic cell motility of neutrophils. No effect of endotoxin on neutrophil migration was observed in their *in vitro* systems, but rotation of the cell-endotoxin mixture was not carried out and incubation of the blood-endotoxin mixture was irregular, again making it unlikely that optimum conditions for *in vitro* phagocytosis were provided. Failure to demonstrate an effect *in vitro* led these authors to regard the interaction between neutrophils and endotoxin as an indirect one mediated by *in vivo* mechanisms.

The present studies as well as those of Martin and Chauduri demonstrate that endotoxin does indeed modify neutrophil migration *in vitro*.[11] Moreover, this interaction causes neutrophil degranulation and decreases the motility of individual non-aggregated neutrophils. Thus, while it would appear that the intact animal provides a much more efficient milieu for the interaction of neutrophils and particulate material, similar results are obtained when these conditions are approached *in vitro*.

There are several other lines of evidence suggesting that leukocyte injury by endotoxin occurs as a result of phagocytosis. Cohn and Morse observed that incubation with either endotoxin or bacteria enhanced the ability of neutrophils to ingest and kill a second bacterial challenge.[13] These authors found that endotoxin and phagocytosis of bacteria produced similar increases in lactic acid production and glucose utilization but were unlike in one respect—only ingestion of bacteria was associated with in-

creased neutrophil oxygen uptake. However, similar studies by Strauss and Stetson did demonstrate increased oxygen uptake following exposure of neutrophils to endotoxin, latex particles, or antigen-antibody complexes.[14] Furthermore, studies by Kerby and by Ribble have demonstrated endotoxin-induced release of lysozyme from neutrophils both *in vitro* and *in vivo*.[15,16] This enzyme is a constituent of neutrophil granules (lysosomes)[17] which are lysed in response to phagocytosis, providing further evidence that endotoxin is ingested by neutrophils. Direct morphologic evidence of phagocytosis of endotoxin by neutrophils has been presented by Mesrobeanu.[18]

Recent evidence suggests that the effect of antigen and antibody on neutrophils may represent yet another instance of neutrophil response to phagocytosis. Uriuhara observed that neutrophils from an Arthus site and non-immune neutrophils incubated *in vitro* with antigen-antibody complexes contained these complexes, some of which were in phagocytic vacuoles.[19] Fusion of the involved phagocytic vacuoles with neutrophil granules (lysosomes) was also observed, resembling events thought to subserve the intracellular digestion of bacteria. Miescher has demonstrated agglutination of both neutrophils and platelets by immune complexes *in vitro*.[20]

The use of relatively impure endotoxin in these experiments requires some discussion of its relationship to "purified" endotoxin. Several lines of evidence suggest that endotoxin toxicity is dependent on its particulate components. These components can be sedimented by high speed centrifugation and appear to correlate with the presence of rod-like forms visualized with the electron microscopy.[21,22] These forms are estimated to be 200-600 Å long and 50-60 Å wide and to possess a molecular weight of approximately 500,000 to 1,500,000.[22] Toxicity and pyrogenicity correlate to some degree with the size of these elements.[21,22] Dispersion into smaller sub-units by sodium desoxycholate, by sodium lauryl sulfate, or by incubation of endotoxin in plasma, is associated with loss of endotoxin pyrogenicity.[22,23] Removal of sodium desoxycholate, or addition of pronase and ethanol to endotoxin inactivated by plasma, restores endotoxin pyrogenicity in association with reappearance of larger rod-like structures.[23,24] The toxic, rod-like structures are thought to represent polymers of smaller non-toxic units.

The fundamental toxic unit of endotoxin has not been isolated in pure form and most endotoxin preparations contain a variety of non-toxic materials.[21,22] Beer and co-workers found approximately 50 per cent of their endotoxin was serologically reactive but non-toxic.[25] These authors were able to concentrate toxic components of endotoxin by differential sucrose

density centrifugation but found this material was still quite heterogeneous morphologically.[21]

Chromatographic separation of endotoxin components provides further evidence of endotoxin heterogenicity.[28] Chromatographic fractions having variable chemical composition produce different degrees of biologic activity in toxicity studies, in Shwartzman skin tests, and in enhancement of non-specific resistance. Endotoxin preparations, therefore, must be regarded as a heterogeneous mixture of cell wall components which have widely varying biologic activity depending on the conditions of endotoxin formation and the indicator system used for endotoxin analysis. Purification attempts have yielded endotoxin preparations that are only relatively homogeneous with respect to particle size and still require particulate materials for biologic activity.[21,22]

The present study suggests that neutrophils are susceptible to cell injury by larger and, therefore, sedimentable fractions of bacterial endotoxin and that changes in leukocyte function during endotoxemia may reflect phagocytosis of endotoxin particles by human neutrophils. The similarity of the human neutrophil reaction to endotoxin, antigen-antibody complexes, bacteria, and latex particles may in part explain the similarity of the inflammatory response to different types of cell injury.

All of these cell-particle interactions may have consequences such as cellular aggregation and release of physiologically-active intracellular factors that would favor localization and irradication of microbial infection. Such a sequence may be continually evolving at a slow rate to remove blood stream contaminants. Such normal clearance mechanisms may participate in the pathogenesis of the signs of overwhelming infection such as shock, intravascular coagulation, leukopenia, and thrombocytopenia.

SUMMARY

Incubation of blood with bacterial endotoxin causes neutrophil aggregation and decreased neutrophil migration in capillary tubes. These effects have been attributed to phagocytosis of endotoxin on the basis of the following evidence: 1.) Similar changes are produced by phagocytosis of a variety of particles. 2.) Removal of heat-labile plasma factors, divalent cation chelation, and low temperatures affect the endotoxin-neutrophil interaction in a manner suggesting a phagocytic event. 3.) Endotoxin suspensions cause degranulation of neutrophils.

Antigen-antibody interactions cause similar changes in leukocytes. It is suggested that neutrophil aggregation, inhibition of neutrophil migration, and neutrophil degranulation represent a general pattern of response to particle ingestion.

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