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ALTERATIONS IN TYPE AND BACTERICIDAL ACTIVITY OF MOUSE PERITONEAL PHAGOCYTES AFTER INTRAPERITONEAL ADMINISTRATION OF ENDOTOXIN

by

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The constant migration of leukocytes through the gingival pocket epithelium has never been studied in detail as a factor in host resistance to bacterial infection, although its possible importance in this respect has been recognized. As early as 1916, *Mendel* stated that gingival exudates contained numerous leukocytes. Recently, *Sharry & Krasse* (1960) demonstrated that the gingival sulcus was the major site of entrance of leukocytes into the oral cavity. The mucous layer covering the gingiva provides an excellent environment for these leukocytes and they are often seen filled with ingested microorganisms indicating that phagocytosis takes place *in vivo* and that the leukocytes may represent an active defense mechanism in the oral cavity (*Rovelstad* 1960, *Egelberg* 1963). *Klinkhamer* (1963) has presented evidence that extremely large numbers of leukocytes can pass through the pocket epithelium into the oral cavity, and both *Rovelstad* (1960) and *Klinkhamer* (1963) have shown that such leukocytes exhibit phagocytic activity *in vitro*.

In view of the fact that bacterial endotoxins are able to affect both the cellular and humoral defense mechanism of the host (*Bohme* 1958; *Shilo* 1959), it was thought of considerable interest to investigate the influence of endotoxin from oral gram-negative bacteria on the activity of phagocytic cells, as these endotoxins probably are released by bacterial autolysis in the gingival crevice and may exert their influence on tissues (*Jensen & Mergenhagen* 1964) and leukocytes in this environment. In the present study, we have tried to evaluate the effect of intraperitoneal injections of bacterial endotoxins on the bactericidal activity of the phagocytic cells residing in the peritoneal cavity of the mouse. The peritoneal cavity offers a convenient biological model for the study of phagocytic processes (*Whitby & Rowley* 1959, *Rowley* 1960, *Jenkin & Palmer* 1960). Furthermore the peritoneal cavity of the mouse is an excellent source of phagocytic cells for *in vitro* studies (*Rowley & Whitby* 1960, *Jenkin & Benacerraf* 1960, *Cooper & West* 1962).

MATERIALS AND METHODS

Bacteria

Two strains of streptococci designated S-SM and S-EV were isolated from the human gingival sulcus (*Jensen & Mergenhagen* 1964). One strain of *Escherichia coli* designated C-BJ-3 was originally isolated from mouse feces (*Jensen, Mergenhagen, Fitzgerald & Jordan* 1963). One strain of an oral diptheroid organism designated D-732 was kindly supplied by Dr. Arden Howell, National Institute of Dental Research. All strains were maintained in Brain Heart Infusion broth (Difco) and periodically checked for purity by surface-streaking on blood agar plates. All cultures were incubated aerobically.

Endotoxin

Purified lipopolysaccharides (LP) were prepared from two strains of *Fusobacterium polymorphum* designated F-RP and F-B1 (*Araujo, Varah & Mergenhagen* 1963). Lyophilized preparations of endotoxin were solubilized in pyrogen-free saline (Baxter Laboratories) and injected intraperitoneally in 0.1 ml amounts containing 100 μg endotoxin in all cases.

Animals

Female albino mice of the CFW strain weighing 16—20 grams were supplied by the NIH Animal Production Unit and used in all experiments.

Source of Serum and Opsonization of Bacteria

Pooled normal rabbit serum was obtained from adult New Zealand white rabbits. Serum was sterilized by filtration and stored at 5° C. Mouse serum was collected from normal CFW mice, pooled and stored at — 20° C until used. For opsonization used in *in vitro* experiments, 0.5 ml mouse serum was mixed with 0.5 ml of a bacterial suspension containing approximately 3×10^8 organisms/ml and incubated in a waterbath at 37° C for 15 minutes. The mixture was then placed in ice and before inoculation diluted in Eagle's medium and finally in Eagle's medium plus heparin and 10 per cent rabbit serum to contain approximately 3×10^4 organisms/ml.

Peritoneal Phagocytes

Collection of phagocytic cells from the peritoneal cavity of mice and infection of the phagocytes with bacteria were performed in two different ways (*Whitby & Rowley* 1959, *Jenkin & Benacerraf* 1960).

In the first type of experiment, saline suspensions of bacteria were injected intraperitoneally in volumes of 0.2 ml containing 10^5 or 10^6 viable bacteria. After 10—15 minutes, the mice were sacrificed by cervical dislocation. Chest and abdomen were sterilized with 95 per cent alcohol and iodine and 2.0 ml of Eagle's basal medium containing 0.5 International Units (I.U.) of heparin per ml and 10 per cent normal rabbit serum was injected into the peritoneal cavity. After gentle massage of the abdomen to obtain a mixture of bacteria and phagocytic cells, the skin was reflected and 1.0 to 1.5 ml of peritoneal washout was collected through a small abdominal incision. Pooled peritoneal washouts were dispensed in 2.0 or 2.5 ml volumes in 1×10 cm tubes, incubated in a waterbath at 37° C and agitated mechanically. To evaluate the phagocytic activity in the peritoneal washouts while incubated *in vitro*, 0.2 ml samples were taken im-

mediately after collection and after one and two hours incubation. The samples were diluted by addition of 1.8 ml cold sterile saline, ground in a motor-driven tissue grinder with a teflon pestle at high speed for one minute, and 0.1 ml of appropriate dilutions were surface-streaked on duplicate blood agar plates. When the phagocytic process was separated into the ingestion phase and intracellular killing phase, duplicate samples of 0.2 ml peritoneal washouts were taken at the same times as before and both samples diluted 1: 10 in cold sterile saline. One sample was treated as before and spread on duplicate blood agar plates to enumerate total viable bacteria present. The other sample was centrifuged at 500 RPM for 5 minutes to sediment phagocytic cells and leave free bacteria in suspension. Supernatants were carefully decanted and aliquots surface-streaked on blood agar. The pellet of phagocytic cells containing attached or fully ingested bacteria was resuspended in 2.0 ml of saline, homogenized in the tissue grinder and aliquots were spread on duplicate blood agar plates. This method described by *Cohn & Morse* (1959) supplies information on total viable bacteria, free bacteria, and bacteria associated with phagocytic cells at each sampling time.

In the second type of experiment, bacteria were not injected into the peritoneal cavity, but phagocytic cells were collected as before. Pooled peritoneal washouts were dispensed in Porter flasks (Kontes Glass Co., New Jersey) in 1.0 ml volumes and incubated for one hour at 37° C. After this period phagocytic cells had settled to the bottom and formed a thin film. The supernatants were discarded and 1.0 ml of a bacterial suspension in Eagle's medium plus 0.5 I.U. heparin/ml and 10 per cent normal rabbit serum was added to each flask. The bacterial inoculum contained in all experiments approximately 3×10^4 viable organisms per ml. Three to four control flasks without phagocytic cells were inoculated at the same time as the experimental flasks and all flasks were incubated for one hour. At this time all supernatants were decanted and appropriate dilutions plated on duplicate blood agar plates to enumerate free bacteria. The cell layers in each flask containing attached or fully ingested bacteria were washed twice with cold sterile saline and the cells then suspended in 2 ml of cold saline by vigorous shaking and

scraping of the bottom. The flasks were placed in an ice bath for 10—15 minutes, shaken again and the contents ground in a tissue grinder. Dilutions were plated on duplicate blood agar plates. Calculations of phagocytosis and bactericidal activity were performed in a similar manner to that described by *Jenkin & Benacerraf* (1960). The difference (D) between the numbers of bacteria in the supernatants of the control flasks (C) and of the experimental flasks (E) would indicate the degree of phagocytosis (C—E=D), while the number of viable bacteria recovered from the layer of phagocytic cells (S) would show to what extent ingested bacteria were killed within the cells. Some of the bacteria recovered from the cell layer might not represent truly ingested bacteria but only organisms attached to the surface of phagocytic cells. Nevertheless, differences in survival of bacteria in the cell layers derived from normal and endotoxin-treated mice in the same experiment would represent true differences in bactericidal activity of the cells. Per cent ingestion and intracellular survival of bacteria can be calculated respectively as $\frac{D \times 100}{C}$ and $\frac{S \times 100}{D}$.

Counts on Peritoneal Phagocytes

The number of phagocytic cells in peritoneal washouts was counted in a Neubauer hemocytometer at 430 × magnification. Tyrode's balanced salt solution without colored indicator was used for collection of washouts to facilitate counting procedures. All values given represent averages of duplicate counts. Differential counts were done on Giemsa stained preparations of 0.02 ml peritoneal washouts spread over a circular area with a diameter of 1.0 cm, using an oil immersion lens.

RESULTS

The Effect of Endotoxin on the Bactericidal Activity of Mouse Peritoneal Phagocytes as Measured Partially *In Vivo* and *In Vitro*

In a previous publication (*Jensen & Mergenhagen* 1964) we have shown that the clearance of viable oral streptococci from the peritoneal cavity of mice could be enhanced by pretreatment with fusobacterium endotoxin administered 48 hours before intra-

Table 1.

Influence of endotoxin administration 48 hours before intraperitoneal infection with oral streptococci (strain S-SM) on bactericidal activity in pooled peritoneal washouts incubated in vitro.

Sampling Time	Exp. 1 Infecting dose: 10^5 oral streptococci		Exp. 2 Infecting dose: 10^6 oral streptococci		Exp. 3 Infecting dose: 10^6 oral streptococci	
	Bact./ml peritoneal washout		Bact./ml peritoneal washout		Bact./ml peritoneal washout	
	Endotoxin*) Mice	Normal Mice	Endotoxin Mice	Normal Mice	Endotoxin Mice	Normal Mice
0 Hr.	43,200	57,700	373,000	438,000	345,000	283,000
1 Hr.	11,500	47,600	163,000	—	97,000	162,000
2 Hr.	11,400	47,200	90,000	342,000	94,000	180,000
Per cent Reduction	73	17	75	22	72	36

*) 100 μg F-RP-LP injected intraperitoneally

Table 2.

Influence of endotoxin administration 3 hours and 48 hours before intraperitoneal infection with 1—2×10^5 oral streptococci on bactericidal activity in pooled peritoneal washouts incubated in vitro.

Pretreatment of mice	Time of Sampling	Exp. 1 Strain S-EV	Exp. 2 Strain S-EV	Exp. 3 Strain S-SM
Endotoxin*) 3 hours before infection	0 Hr.	105,000**)	46,400	20,100
	1 Hr.	114,000	49,400	21,600
	Per cent Reduction	None	None	None
Endotoxin 48 hours before infection	0 Hr.	65,000	45,900	21,000
	1 Hr.	18,300	9,200	5,100
	Per cent Reduction	72	80	76
Saline	0 Hr.	55,300	37,800	18,400
	1 Hr.	36,700	19,000	10,400
	Per cent Reduction	34	50	44

*) 100 μg F-RP-LP injected intraperitoneally
**) No. bacteria/ml peritoneal washout

peritoneal challenge with bacteria. This effect was ascribed to the stimulating influence of endotoxin on the phagocytic processes in the peritoneal cavity (*Whitby & Rowley* 1959, *Rowley* 1960, *Jenkin & Palmer* 1960).

To further establish the major role of phagocytic cells normally residing in the peritoneal cavity of the mouse in the clearance of oral bacteria from this area it was decided to break into the processes going on within the peritoneal cavity after infection with streptococci and study further events *in vitro*. To this end the method described by *Whitby & Rowley* (1959) was utilized. One group of mice was injected intraperitoneally with 100 μg fusobacterium endotoxin while another group received the same volume of pyrogen-free saline to serve as a control. Forty-eight hours later when the endotoxin, according to previous experiments, had stimulated the resistance of the pretreated mice, both groups were injected intraperitoneally with 1×10^5 viable oral streptococci. After 10 minutes the mice were sacrificed, peritoneal washouts collected and pooled from 2 mice within each group. The washouts containing a mixture of bacteria and phagocytic cells were incubated in a waterbath at 37° C for two hours. Samples for enumeration of viable streptococci in the washout were collected and prepared as previously described. Any reduction in viable count during the period of incubation would indicate phagocytosis and destruction of bacteria in the closed system.

Representative examples from several similar experiments are shown in Table 1. Streptococci have been injected in two different dosages but within these limits the number of bacteria administered intraperitoneally does not influence the degree of phagocytosis. In all cases the reduction in numbers of viable bacteria is far greater in peritoneal washouts from endotoxin-treated mice than from normal mice, indicating an increased phagocytic capacity. The stimulating effect of fusobacterium endotoxin on the clearance of streptococci from the peritoneal cavity of mice thus could be correlated to an increase in phagocytic activity in the peritoneum.

The same type of experiment was performed utilizing two different strains of oral streptococci for intraperitoneal infection in normal mice and mice pretreated with 100 μg fusobac-

terium endotoxin respectively 48 hours and 3 hours before bacterial challenge. The results are shown in Table 2. The reduction in viable bacteria during one hour incubation *in vitro* was 72—80 per cent in peritoneal washouts from mice pretreated with endotoxin 48 hours previously, as compared to a reduction of 34—50 per cent in washouts from normal mice. No reduction in viable counts was observed in peritoneal washouts from mice pretreated with endotoxin 3 hours before injection of bacteria indicating a considerable decrease in phagocytic activity in the exudates. The biphasic effect of endotoxin on the host defense mechanism characterized by an initial depression and subsequent stimulation of resistance to bacterial infection is quite evident.

Further studies of the phagocytic processes in similar combined *in vivo* and *in vitro* experiments utilizing the previously described technique to distinguish between the ingestion phase and the phase of intracellular destruction of streptococci by the phagocytic cells in peritoneal washouts revealed, however, that only the second phase of phagocytosis was measured in this type of experiment (Fig. 1). The number of free streptococci in pe-

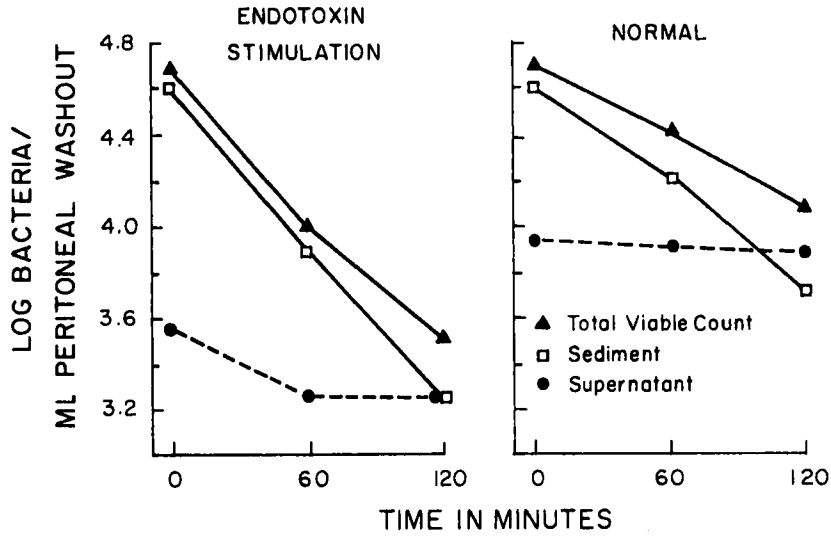

Fig. 1. *In vitro* interaction between mouse peritoneal phagocytes and oral streptococci in peritoneal washouts from normal mice and mice pretreated with 100 μg endotoxin 43 hours earlier.

ritoneal washouts remained for all practical purposes constant during 2 hours incubation *in vitro,* and the decrease in total viable count observed during the same period was due to killing of bacteria already located intracellularly. Obviously ingestion of these bacteria must have taken place intraperitoneally in the time interval between injection of bacteria and collection of peritoneal washouts. The differences observed in phagocytic activity in peritoneal washouts from endotoxin-treated and normal mice *in vitro* then actually is a difference in intracellular destruction of bacteria.

The Effect of Endotoxin on Number and Types of Phagocytic Cells in the Peritoneal Cavity of the Mouse

It was noted that fusobacterium endotoxin, when injected intraperitoneally in mice, greatly affected the number of phagocytic cells recovered in peritoneal washouts collected subsequently. While washouts from normal mice generally contained from 2,000—3,000 phagocytic cells per mm^3, washouts collected 3 hours after endotoxin administration would contain only $^1/_3$

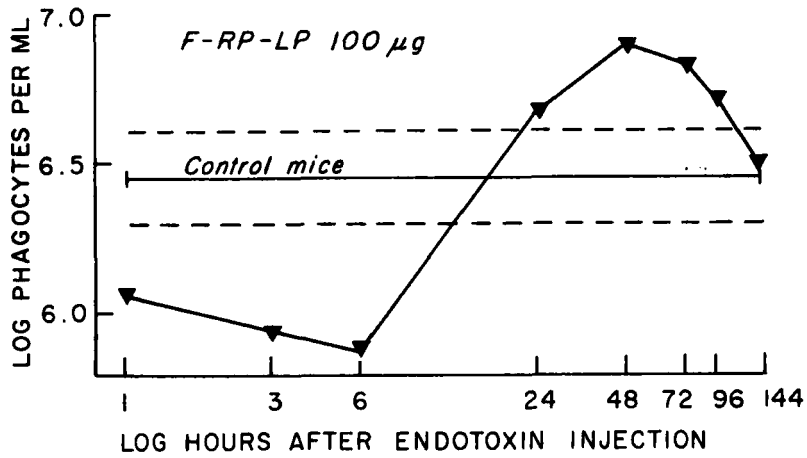

Fig. 2. Changes in numbers of mouse peritoneal phagocytes following intraperitoneal administration of fusobacterium endotoxin (F-RP-LP).

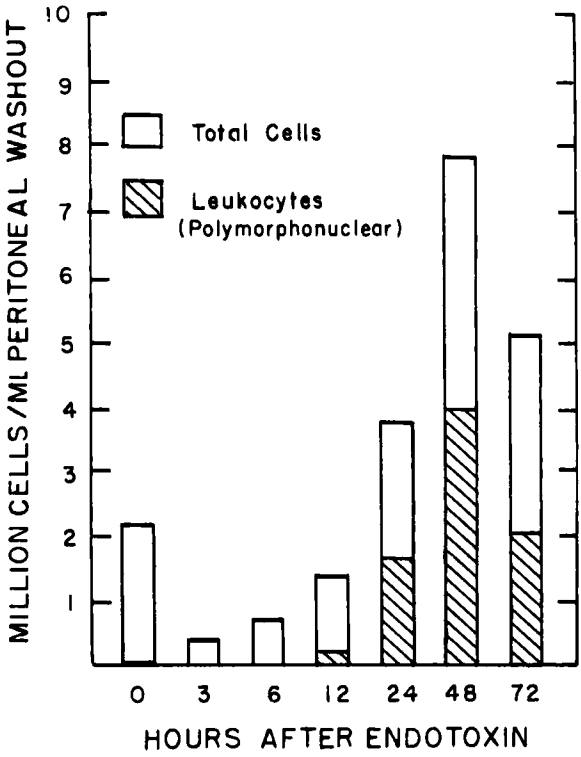

Fig. 3. Changes in numbers and types of mouse peritoneal phagocytes following intraperitoneal administration of 100 μg fusobacterium endotoxin (F-Bl-LP).

of this number, and washouts collected 48 hours after endotoxin injection contained approximately 3 times as many phagocytic cells as washouts from normal mice. Figure 2 shows variations in the number of phagocytic cells in peritoneal washouts collected from mice at different times after intraperitoneal injection of 100 μg fusobacterium endotoxin. The values obtained represent duplicate counts of washouts from groups of 6 to 10 mice. These variations in the number of potentially phagocytic cells in peritoneal exudates from endotoxin-treated mice could at least partly explain the previously described variations in phagocytic capacity.

The results from a second experiment utilizing another preparation of fusobacterium endotoxin (F-B1-LP) for intraperi-

toneal injection are shown in Fig. 3. The columns represent average of total cell counts in peritoneal washouts from groups of 8 to 11 mice and show the same variations in number of peritoneal phagocytes after endotoxin administration as before. The shaded portions of the columns indicate the number of polymorphonuclear cells present in the washout while the unshaded portions represent the number of mononuclear cells. These values are based on the ratio between the differential count in Giemsa stained smears and the total count in the same washouts. The major part of phagocytic cells in the peritoneal cavity of normal mice are large mononuclear cells often termed macrophages and only very few polymorphonuclear cells are present. Twelve and 24 hours after endotoxin is administered intraperitoneally an increasing amount of polymorphonuclear forms are found in peritoneal exudates, and at 48 hours 50 per cent of the cells present in the washouts are polymorphonuclear. The decrease in cell numbers observed a few hours after endotoxin administration seem to be due to a decrease in the original mononuclear cell population while the increase in total cell numbers seen later to a large extent is caused by an influx of polymorphonuclear leukocytes. The mononuclear cell population has returned to normal size 24 hours after endotoxin administration and attains values somewhat above normal at 48 and 72 hours.

Variations in the number of cells as well as alterations of the cell types present in the peritoneal cavity of mice after intraperitoneal injection of fusobacterium endotoxin most certainly could affect the outcome of the phagocytic processes studied *in vitro*. These possibilities were considered in the next series of experiments.

The Effect of Pretreatment with Endotoxin on the Bactericidal Activity of Mouse Pertioneal Phagocytes *In Vitro*

To obtain evidence for the possible effect of fusobacterium endotoxin on ingestion as well as intracellular killing of bacteria by peritoneal phagocytes it was necessary to use a complete *in vitro* system as described by *Rowley & Whitby* (1959) and *Jenkin & Benacerraf* (1960). Peritoneal washouts were collected

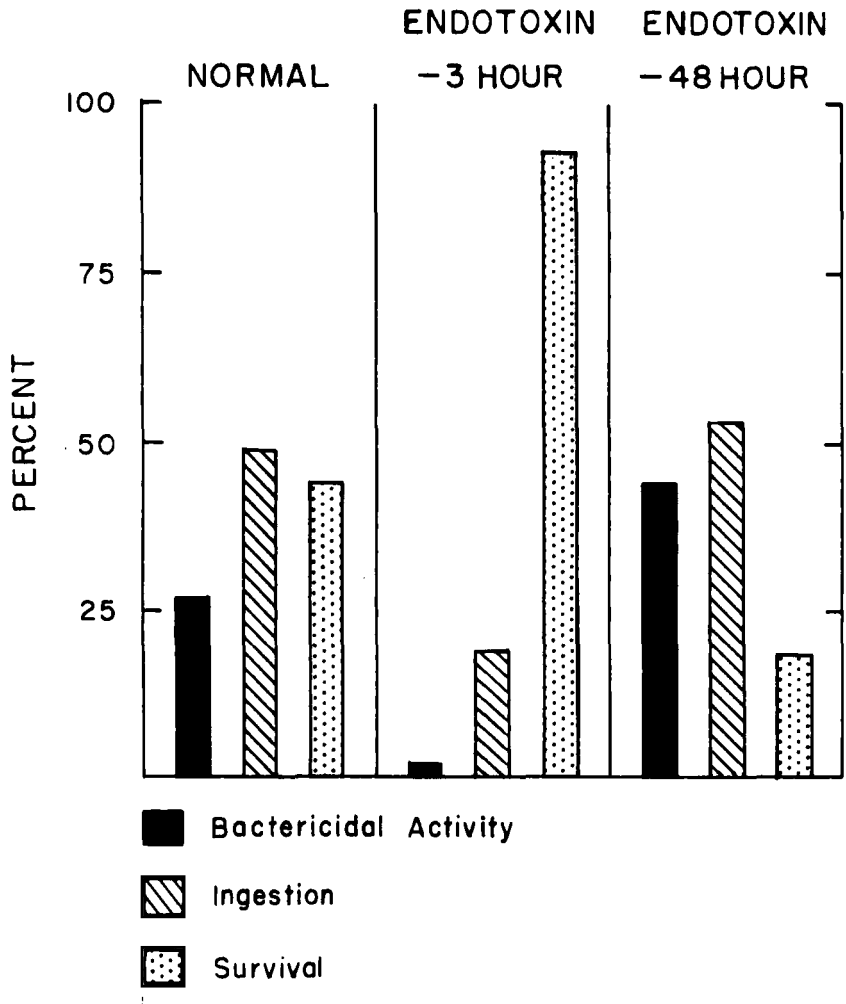

Fig. 4. *In vitro* interaction between *Escherichia coli* and peritoneal phagocytes from normal mice and mice pretreated with 100 μg fusobacterium endotoxin. No equilibration of cell numbers. Total bactericidal activity, ingestion and intracellular survival are illustrated.

from mice injected intraperitoneally with 100 μg fusobacterium endotoxin 3 hours and 48 hours earlier, and from normal mice. Washouts were pooled from 4 mice within each group, dispensed in 1.0 ml amounts in Porter flasks and incubated for one hour at 37° C to allow the phagocytic cells to settle and form a layer

on the bottom. The supernatants were then discarded and all flasks inoculated with 1.0 ml of a suspension containing approximately 30,000 *E. coli*/ml as the test organism. Ingestion and intracellular survival of bacteria were measured after one hour incubation as previously described. The results shown in Fig. 4 represent for each group, averages of bactericidal activity in four flasks. The influence of endotoxin on the bactericidal activity in mouse peritoneal washouts is also evident in this type of experiment where ingestion as well as intracellular killing of bacteria take place *in vitro*. However, it is interesting to note that while both ingestion and intracellular killing are depressed in washouts from mice pretreated with endotoxin 3 hours before collection, the main difference between the activity in washouts from normal mice and mice pretreated with endotoxin 48 hours earlier is a difference in the destruction of intracellular located bacteria.

As mentioned previously the differences in phagocytic activity in washouts from normal and endotoxin-treated mice might be due to differences in the number of phagocytically-active cells harvested from the peritoneal cavity of these mice. The experiment was therefore repeated but included steps to insure that cell suspensions from the three experimental groups contained the same number of phagocytic cells. Peritoneal washouts were collected and pooled from 8—10 mice in each group and after initial cell counts were centrifuged at 500 RPM for 5 minutes to sediment the phagocytic cells. Supernatants were discarded and the cells resuspended in fresh medium to contain approximately 2,000 phagocytes per mm^3 for all three experimental groups. Staining with 1 per cent trypan blue showed that 90—96 per cent of the cells were viable after this procedure. Cell suspensions from each group of mice were distributed in 1.0 ml amounts to six Porter flasks, and the experiment continued as described earlier. The inoculum was 35,000 *E. coli* per flask.

The adjustment of cell numbers in suspensions of peritoneal phagocytes from normal and endotoxin-treated mice affects the results considerably (Fig. 5). It is now evident that the phagocytic capacity of peritoneal cells from mice pretreated with endotoxin 3 hours before collection is approximately equal to the capacity of phagocytic cells from normal mice. This suggests

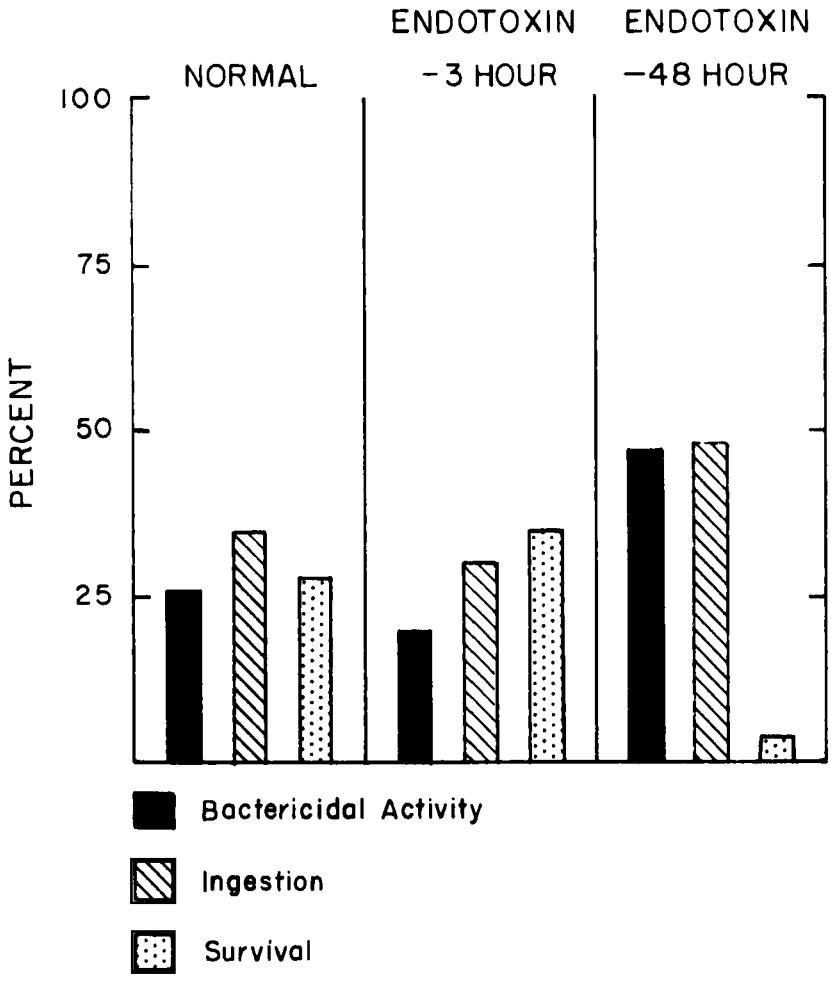

Fig. 5. *In vitro* interaction between *Escherichia coli* and peritoneal phagocytes from normal mice and mice pretreated with 100 μg fusobacterium endotoxin. Concentration of peritoneal phagocytes adjusted to 2×10^6/ml in all flasks. Total bactericidal activity, ingestion and intracellular survival are illustrated.

that the decrease in bactericidal activity observed in unequilibrated peritoneal washouts from mice 3 hours after endotoxin administration (Table 2) is mainly, if not completely, due to a decrease in the number of phagocytically-active cells in the peritoneal cavity at this time. On the other hand, suspensions of

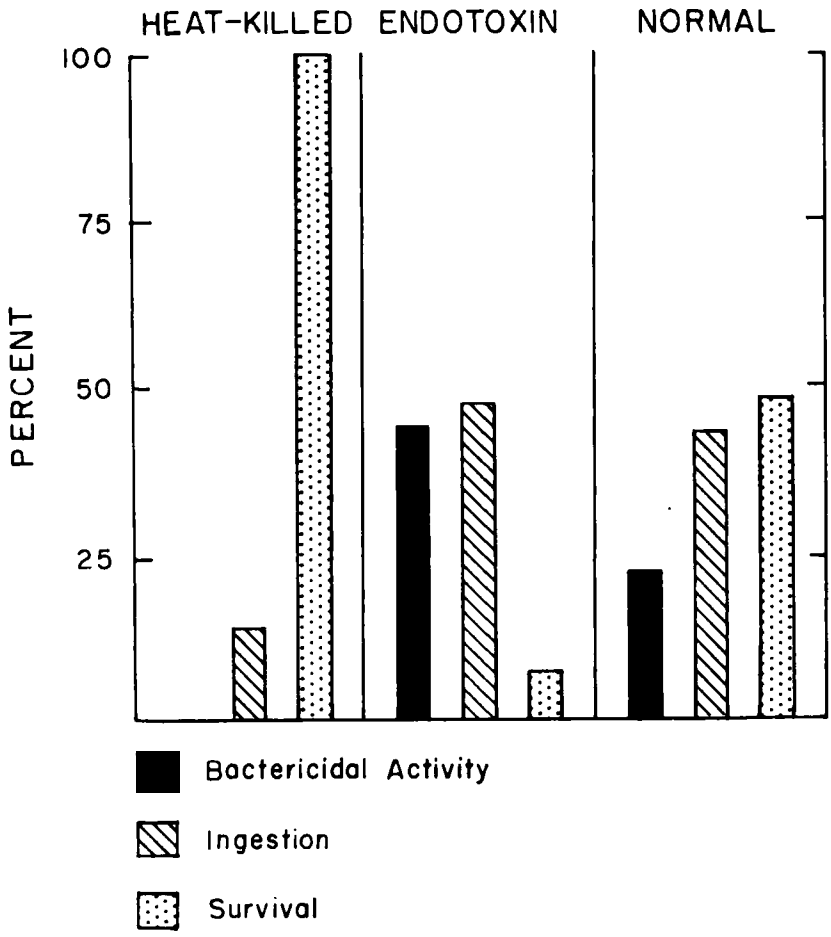

Fig. 6. *In vitro* interaction between *Escherichia coli* and peritoneal phagocytes from normal mice and mice pretreated with 100 μg fusobacterium endotoxin 48 hours earlier. Concentration of peritoneal phagocytes adjusted to approximately 2×10^6/ml. Heat-killed phagocytic cells used as control. Total bactericidal activity, ingestion and intracellular survival are illustrated.

peritoneal phagocytes collected from mice pretreated with endotoxins 48 hours earlier still show a definite increase in bactericidal activity after adjustment of cell numbers. Although the capacity for ingestion of bacteria appears to be somewhat enhanced in these cells, the most striking difference between peritoneal phagocytes from normal and endotoxin-stimulated mice

is found in their capacity for intracellular destruction of bacteria.

With the method employed to separate the ingestion phase from the killing phase by phagocytes, there remains the possibility that some bacteria disappear from the supernatants of inoculated flasks simply by physical adhesion to the bottom layer of phagocytic cells and not due to any active phagocytic process. In this case the values obtained would not indicate true phagocytosis. To investigate this possibility, experiments were repeated utilizing as control Porter flasks containing phagocytic cells from normal mice. These flasks were heated at 100° C in a waterbath for 10 minutes to kill the cells attached to the bottom. Cells in washouts from normal mice and mice injected with endotoxin 48 hours previously were adjusted to the same number as before and all flasks inoculated with 33,000 *E. coli*. As seen in Fig. 6, thirteen per cent of viable bacteria had disappeared from the supernatants after one hour incubation in control flasks containing heat-killed phagocytes, but the total inoculum could be recovered from the cell-layer by washing. In the experimental flasks, containing live peritoneal phagocytes, considerably more bacteria disappeared from the supernatants indicating active phagocytosis, and only part of these ingested (or attached) bacteria could be recovered from the cell-layer. The significant difference in survival of bacteria in cell-layers from normal mice and endotoxin-treated mice was again evident indicating an increased capacity for destruction of bacteria ingested by the latter cell population.

In the *in vitro* experiments just described a strain of *E. coli* was used as a convenient test organism. The same experiments have been repeated several times using as inoculum two different strains of oral streptococci and an oral diphtheroid organism to determine if the increase in bactericidal activity observed in phagocytic cell populations from the peritoneal cavity of mice pretreated with endotoxin 48 hours earlier was limited

Fig. 7. *In vitro* bactericidal activity of peritoneal phagocytes collected from normal mice and mice pretreated with 100 μg fusobacterium endotoxin 48 hours earlier. *Escherichia coli*, an oral diphtheroid organism and an oral streptococcus are used as test bacteria. Concentration of peritoneal phagocytes adjusted to 2×10^6/ml. Total bactericidal activity, ingestion and intracellular survival are illustrated.

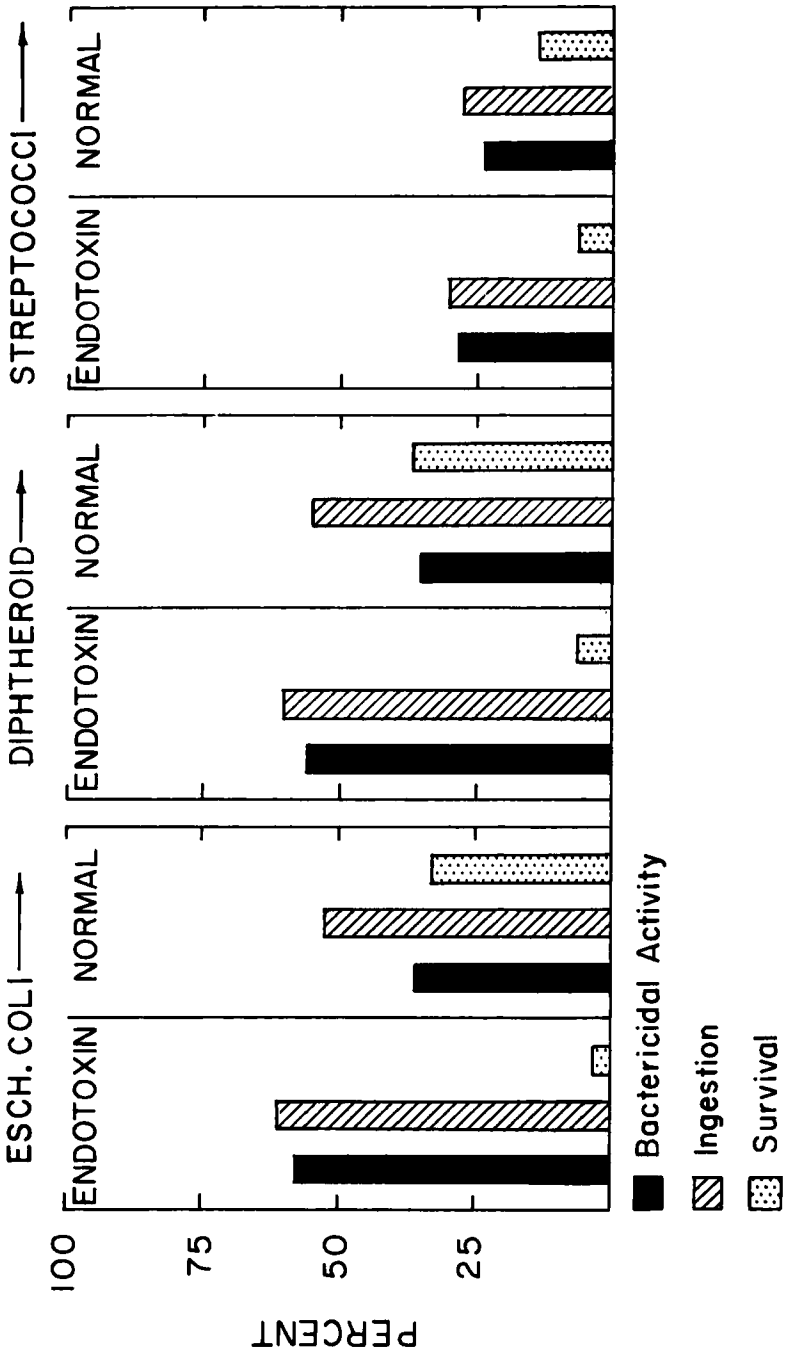
ESCH. COLI
DIPHTHEROID
STREPTOCOCCI
ENDOTOXIN
NORMAL
ENDOTOXIN
NORMAL
ENDOTOXIN
NORMAL
PERCENT
100
75
50
25
Bactericidal Activity
Ingestion
Survival

Fig. 7.

to gram-negative bacteria. The results are shown in Fig. 7. The values presented are averages of phagocytic activity in 6 experimental flasks per group, and the number of phagocytic cells in the suspension have been adjusted to the same size, approximately 2,000 cells/mm^3. The inocula used were approximately 40,000 *E. coli,* 30,000 diphtheroids and 35,000 streptococci. Regardless of the bacterial species utilized as test organism, there is always observed a greater bactericidal activity in flasks containing phagocyte populations from endotoxin-treated mice than in flasks with peritoneal phagocytes from normal mice. This appears to be partly due to a slightly enhanced capacity for ingestion of bacteria but mainly to a pronounced increase in nonspecific intracellular destruction of ingested bacteria. It should be noted, however, that oral streptococci seem to be more resistant to phagocytosis by mouse peritoneal phagocytes than the two other bacterial species, when estimated in this system.

DISCUSSION

Injection of minute quantities of lipopolysaccharide endotoxins derived from oral fusobacteria into the peritoneal cavity of the mouse exert a profound influence on the phagocytic activities in this region as measured by experiments performed partly *in vivo* and partly *in vitro* or exclusively *in vitro.* Three hours after endotoxin administration the phagocytic activity in peritoneal washouts is appreciably depressed whereas an increase is observed in washouts collected 48 hours later. This initial decrease and subsequent increase in phagocytic activity closely parallels the well-known biphasic effect of bacterial endotoxins on the host defense mechanism against bacterial infection (*Shilo* 1959). The first phase of decreased phagocytic activity seems exclusively to be caused by a decrease in the numbers of phagocytically-active cells in the mouse peritoneal cavity as the remaining cells are just as capable of phagocytosis as cells from normal mice. The decrease in cell number is probably due to the cytotoxic effect of endotoxin and many partially destroyed cells are observed in peritoneal washouts collected at this time. The increase in phagocytic activity observed in washouts from endotoxin-treated mice in the later phase can also to a certain extent

be explained by alterations in cell numbers and the presence of an increased number of phagocytes in the peritoneal cavity. On the other hand, the phagocytic capacity of such cells, when compared to an equal number of peritoneal cells from normal mice, is still enhanced. More importantly, the capacity for intracellular destruction of bacteria is considerably increased. This might be due to the concomitant alterations in the composition of the phagocytic cell population. While peritoneal washouts from normal mice contain 96—98 per cent mononuclear cells, approximately 50 per cent of the cell-population in washouts from mice injected with endotoxin 48 hours earlier are polymorphonuclear leukocytes. *Fruhman* (1959) observed a similar influx of polymorphonuclear leukocytes in the peritoneal cavity of rats injected with bacterial endotoxin. Polymorphonuclear leukocytes are generally considered to be very effective phagocytes and are probably further stimulated by endotoxin (*Fritze & Wendt* 1955, *Cohn & Morse* 1960).

However, the mononuclear cell population is probably of considerable importance. *Rowley* (1960), *Jenkin & Palmer* (1960) and *Jenkin & Benacerraf* (1960) reported that intravenous injection of bacterial endotoxins in mice promoted the phagocytic abilities of peritoneal mononuclear macrophages and *Rowley* (1960) found that treatment with endotoxin *in vitro* likewise increased the phagocytic ability of these cells. Further, *Perkins, Marcus, Gyi & Niya* (1958) observed that endotoxin injections specifically enhanced intracellular digestion of chicken erythrocytes by mouse peritoneal macrophages.

The results of the present investigation are of further interest in connection with a recent report by *Auzins & Rowley* (1962) in which they found that peritoneal macrophages from *E. coli* endotoxin-treated mice have markedly increased capacity for intracellular digestion of two antigenically dissimilar strains of enterobacteriaceae. Our results extend these observations in that leukocytes, either polymorphonuclear leukocytes or macrophages, from *F. polymorphum* endotoxin-treated mice have increased capacity for intracellular digestion of gram-positive streptococci or diphtheroids as well as a gram-negative *E. coli.*

The increased phagocytic activity, in particular the intracellular destruction of bacteria by phagocytes, observed in the peri-

toneal cavity of the mouse after intraperitoneal endotoxin stimulation thus seems to depend on a complex of different factors: (1) An increase in the total number of phagocytic cells in the peritoneum, (2) an influx of polymorphonuclear leukocytes, and (3) a mobilization of mononuclear cells from the tissues, or the blood stream to replace the initial decrease in the resident mononuclear cell population.

The observed nonspecific effects of endotoxin on the phagocytic activity in exudates from the mouse peritoneal cavity is only one aspect of the complex mechanism by which endotoxins affect host resistance to bacterial infection. Humoral factors such as an increase in opsonic serum titre (*Jenkin & Palmer* 1960) or an increase in specific bactericidal antibody levels encountered in the serum of endotoxin-treated mice (*Whitby, Michael, Woods & Landy* 1961) are undoubtedly operative in the peritoneal cavity during the phagocytic process.

In view of the fact that large numbers of phagocytically-active leukocytes migrate through the human gingival crevice epithelium and represent part of the local defense mechanism in this area (*Rovelstad* 1960, *Klinkhamer* 1963), the influence of endotoxins liberated from gram-negative bacteria in the gingival crevice on the number and phagocytic properties of these cells might very well be a factor of considerable importance in the development or progression of periodontal disease.

SUMMARY

Intraperitoneal injection of endotoxin from oral fusobacteria produces in mice a biphasic effect on the phagocytic activity in the peritoneal cavity, characterized by an initial depression followed by stimulation. To clarify the underlying mechanism peritoneal exudates were incubated *in vitro* with *Escherichia coli*, oral streptococci, and oral diphtheroid bacilli as test bacteria. The initial depression of phagocytic activity is caused by a decrease in the number of mononuclear phagocytes normally residing in the peritoneal cavity of the mouse, while the subsequent stimulation is due both to an increase in the number of phagocytically-active cells and to an increased phagocytic capacity of the cells present. Such cells show a slightly enhanced

capacity for ingestion of bacteria and a significantly increased capacity for intracellular killing of bacteria. An influx of polymorphonuclear leukocytes is observed in the peritoneal cavity 24 to 48 hours after endotoxin injection. These polymorphonuclear leukocytes may be partly responsible for the increased phagocytic activity observed in the peritoneal exudates.

RÉSUMÉ

MODIFICATIONS DU TYPE ET DE L'ACTIVITÉ BACTÉRICIDE DES PHAGOCYTES DU PÉRITOINE CHEZ LA SOURIS APRÈS ADMINISTRATION INTRA-PÉRITONÉALE D'ENDOTOXINE

L'endotoxine de *Fusobacterium polymorphum* injectée d'une souris par voie intraperitonéale provoque un changement de l'activité phagocytaire dans le peritoine characterisé tout d'abord par une phase de depression suivie d'une phase de stimulation. Les changements de l'activité phagocytaire sont examinés *in vivo* et *in vitro* dans l'exsudat peritonéale incubé de *Escherichia coli* et de souches orales de streptocoques et diphteroides. La depression initiale est causée par une baisse du nombre des cellules mononucleaires, qui se trouvent normalement dans le peritoine de la souris, tandis que la stimulation observée ensuite est causée non seulement par une leucocytose suivante mais aussi par une augmentation du pouvoir phagocytaire des cellules. La capacité de l'englobement phagocytaire des cellules stimulés n'est qu'un peu plus grande que celle des leucocytes normales, mais elles présentent une augmentation significative de leur pouvoir de destruction des bacteries localisée intracellulairement. Les migrations considérables des leucotytes polynucléaires observés dans le peritoine 24—48 heures apres l'injection d'endotoxine sans doute contribuent a la plus grande activité phagocytaire dans la peritoine.

ZUSAMMENFASSUNG

ÄNDERUNGEN IN TYP UND BAKTERIZIDER AKTIVITÄT DER PERITONEALER PHAGOZYTEN IN MÄUSEN NACH INTRAPERITONEALER INJEKTION VON ENDOTOXIN

Intraperitoneale Injektion mit Endotoxin von oralen Fusobakterien ruft in Mäusen einen zweiphasigen Effekt auf die phagozytäre Aktivität in der Peritonealhöhle hervor. Um den zugrun-

deliegenden Mechanismus näher zu untersuchen wurden peritoneale Exsudaten in vitro mit *Escherichia coli,* oralen Streptokokken und oralen Diphtheroiden als Testbakterien inkubiert.

Die beobachtete initiale Depression der Phagozytoseaktivität ist von einem Abnehmen der Zahl der mononukleären Phagozyten, die normalerweise in der Peritonealhöhle der Maus anwesend sind, verursacht, während die nachfolgende Stimulation sowohl von einer Zunahme der Zahl der phagozytierenden Zellen als von einer vergrösserten Aktivität der einzelnen Zellen herrührt. Solche Zellen zeigen nur eine leicht gesteigerte Fähigkeit Bakterien aufzunehmen, aber ihre Kapazität schon einverleibte Bakterien zu töten ist signifikant vergrössert.

Eine Migration polymorphonukleärer Leukozyten ist in der Peritonealhöhle 24 bis 48 Stunden nach der Injektion mit Endotoxin observiert. Diese Granulozyten mögen teilweise für die gesteigerte phagozytäre Aktivität verantwortlich sein.

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