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Effect of *Escherichia coli* Alpha-Hemolysin on Human Peripheral Leukocyte Function In Vitro

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To gain further evidence for the role of the *Escherichia coli* alpha-hemolysin in pathogenesis, its in vitro effects on human peripheral leukocyte function were studied. Leukocytes exposed to low doses of alpha-hemolysin responded with a marked chemiluminescence response, indicating activation of oxidative metabolism. This response was time and dose dependent. Pretreatment of leukocytes with doses of alpha-hemolysin at which nearly 80% of the cells survived decreased the ability of the cells to phagocytize bacteria and particles and to undergo chemotaxis. Premature activation of leukocytes and inhibition of phagocytosis and chemotaxis by alpha-hemolysin, if they occur in vivo, would greatly enhance the survival of an invading *E. coli* strain.

Lovell and Rees (16) were the first to describe a filterable hemolysin produced by *Escherichia coli*. Smith (26) termed the molecule alpha-hemolysin (AH) and, along with others, characterized its properties. AH is a protein (24, 25, 28, 37), possibly a lipoprotein (5), which requires calcium for activity (28) and causes a clear zone of hemolysis on blood agar.

The role of AH in pathogenesis is still unknown. However, several recent reports have shown that hemolysin production is more common in *E. coli* isolated from human extraintestinal infections (30 to 60% of strains) than in *E. coli* isolated from normal human feces (7% of strains) (4, 9, 14, 19, 20, 22). Also, hemolytic *E. coli* strains are more virulent for mice (11, 27, 31, 35) and rats (12, 13) than are hemolysin-negative strains or mutants. van den Bosch and co-workers (31) reported that elimination of hemolysin production in nephropathogenic strains results in a loss of virulence. In later work they demonstrated that the loss of a plasmid coding for hemolysin was associated with the loss of nephropathogenicity. Nephropathogenicity could be restored by reintroduction of the plasmid (33). Welch et al. (35) recently demonstrated that adding the DNA sequence encoding for hemolysin to an avirulent nonhemolytic fecal strain of *E. coli* resulted in an increase in virulence for rats. AH itself has been shown to be cytotoxic in vitro for chicken embryo fibroblasts (7) and mouse fibroblasts (5).

In a recent study in our laboratory, we showed that nanogram amounts of AH are cytolytic for human peripheral blood leukocytes in vitro (6). The purpose of this study was to determine whether low concentrations of AH affect leukocyte functions such as activation, phagocytosis, and chemotaxis.

MATERIALS AND METHODS

Bacteria and culture conditions. The hemolytic *E. coli* strain was a clinical isolate from a patient with a urinary tract infection at West Virginia University Hospital, Morgantown. Isolated hemolytic colonies were identified as *E. coli* by Gram stain and biochemical tests (10).

Staphylococcus epidermidis was a laboratory strain obtained from the West Virginia University culture collection. It was grown in 10 ml of Trypticase soy broth (BBL Microbiology Systems) with 0.1% dextrose at 37°C for 18 to 20 h. For phagocytosis assays, the culture was washed twice in Hanks balanced salt solution (HBS), suspended in 1 ml of HBS, and opsonized by the addition of 2 ml of fresh human serum. The suspension was then incubated for 30 min at 37°C, washed once in HBS, and resuspended in HBS.

Preparation, purification, and assay of AH. AH was prepared, purified, and assayed for hemolytic activity as previously described (5, 6). The specific activity was 200,000 50% hemolytic units (HU_{50}) per mg of protein. The medium used for growth and production of *E. coli* hemolysin was the chemically defined medium described by Snyder and Koch (29).

Leukocyte preparation. Peripheral blood was obtained from healthy volunteers by venipuncture. Leukocytes were isolated by dextran sedimentation as previously described (6). The resulting cell suspension was referred to as human peripheral leukocytes (HPL).

Treatment of leukocytes with low doses of AH. Leukocytes were suspended in 40 ml of bovine serum albumin (BSA)-HBS to a concentration of approximately 1×10^6 to 2×10^6 cells per ml. Two HU_{50} of AH per milliliter or an equal volume of sample buffer was added. The cell suspensions were incubated for 30

min in a 37°C shaking water bath, harvested by centrifugation, washed once in cold phosphate-buffered saline, and resuspended in BSA-HBS. Trypan blue was used for determining viability, and the cell suspensions were adjusted so that they contained an equal number of viable cells per unit volume.

CL. Leukocyte chemiluminescence (CL) was measured in a liquid scintillation spectrometer with the refrigeration off and in the out-of-coincidence mode. Leukocytes (10^6/ml) were suspended in 5 ml of 0.1% BSA-HBS with 10^{-7} M luminol and then placed in dark-treated (at least 24 h in the absence of light) siliconized scintillation vials. The vials were counted for 0.2 min. When not being counted, the vials were kept in a 37°C shaking water bath. Background counts were obtained by counting vials with BSA-HBS alone. The background counts were subtracted from the actual counts, and the values were converted to counts per minute.

Measurement of phagocytosis by CL. AH- or buffer-treated HPL (2.5×10^6) were added to siliconized scintillation vials. The volume of each vial was brought up to 5 ml with BSA-HBS containing 10^{-7} M luminol. CL was then measured as described above for 10 min, after which 2.5×10^8 colony-forming units (CFU) of opsonized *S. epidermidis* prepared as described above were added to each vial. Controls included bacteria with no HPL and HPL (buffer treated) with 500 μg of concanavalin A (Sigma Chemical Co.). CL was then measured for an additional 40 min.

Measurement of phagocytosis by direct plate counts. Into sterile plastic vials 10^7 to 10^8 CFU of opsonized *S. epidermidis* and 5×10^6 to 5×10^7 AH- or buffer-treated HPL were added in a total volume of 6 ml of BSA-HBS without dextrose (a 2:1 bacterium-to-leukocyte ratio was always maintained). A third vial contained opsonized bacteria only. The vials were incubated in a 37°C shaking water bath. At time zero and at 60 min, 1.1 ml was withdrawn from each vial. To measure bacterial survival, 0.1 ml of this solution was added to 9.9 ml of water, blended in a Vortex mixer for 1 min to lyse the HPL, and further diluted in water. Samples of each dilution were added to melted Trypticase soy agar (BBL) and then poured onto plates. To measure bacterial attachment, the remaining 1 ml was centrifuged at $250 \times g$ for 10 min. One-tenth milliliter of supernatant fluid was then diluted and plated on Trypticase soy agar as described above.

Measurement of phagocytosis by ingestion of oil red O-lipopolysaccharide particles. Phagocytosis by AH- or buffer-treated HPL was measured spectrophotometrically with oil red O by the method of Stossel (30). A 0.2-ml amount of a prewarmed (37°C) particle suspension was added to 0.8 ml of prewarmed HPL suspension (10^6 to 10^7 cells per ml) in a 10-ml siliconized conical centrifuge tube. The tube was incubated in a 37°C water bath with periodic agitation for 5 min and assayed as described by Stossel (30).

Chemotaxis. Leukocyte chemotaxis was measured by a modification of the method of Ward and Maderazo (34). A modified Boyden chamber with a 5-μm membrane filter was used. Six-tenths milliliter of an AH- or buffer-treated leukocyte cell suspension (3.5×10^6 viable cells per ml) was added to the upper chamber. An equal volume of the chemoattractant was then added to the lower chamber. Zymosan-activated serum (ZAS) was used as the positive chemoattractant, and BSA-HBS was the negative chemoattractant. ZAS was prepared by suspending 5 mg of zymosan A (Sigma) in 1 ml of fresh human serum. The suspension was incubated for 30 min at 37°C, after which it was diluted 1:10 in HBS. The chemotaxis chambers were incubated in a humid chamber at 37°C for 90 min. All test samples were run in triplicate. The filters were then removed and rinsed twice in HBS, fixed in 95% ethanol, and stained in hematoxylin for 2 min. They were then rinsed in distilled water, decolorized in acid alcohol, dehydrated in two changes of 95% ethanol, cleared in xylene, and mounted upside down on microscope slides. Leukocytes migrating through the filter in five high-power fields were counted for each filter.

RESULTS

Activation of leukocytes by AH. To determine the effect of AH on HPL activation, 10^6 cells per ml were exposed to various concentrations of hemolysin, and the CL response was monitored for 20 to 40 min. The results are shown in Fig. 1, 2, and 3 and represent a typical experiment. Two determinations were made for each point, and each experiment was repeated at least twice. HPL exposed to low doses of AH (1 to 2 HU_{50}/ml [Fig. 1]) exhibited marked and immediate CL. With 1 HU_{50}/ml, the CL displayed biphasic time course kinetics, peaking initially after 5 min of exposure, decreasing over the next 10 min, rising again, and peaking after 27 min of exposure. Treatment of HPL with 2 HU_{50}/ml did not result in a biphasic response but rather in a slow rise, with the peak CL occurring at 20 min of exposure followed by a gradual decline.

HPL treated with higher doses of AH (6, 8, and 10 HU_{50}/ml) exhibited different time course kinetics (Fig. 2). The CL rose more rapidly at all three doses; the higher the dose, the more rapid the rise. The level of CL was as much as 10-fold higher than that of HPL treated with 1 to 2 HU_{50} of AH per ml. After the peak CL was reached (5 to 10 min), the level fell off more rapidly than at lower doses. The CL levels of HPL exposed to buffer remained 1 to 3 logs lower than those of AH-exposed HPL.

To obtain a dose-response curve, the peak CL was plotted for each concentration of AH (Fig. 3). The CL response rose in an approximately logarithmic fashion with respect to the dose of AH over a concentration range of 0 to 4 HU_{50}/ml. At higher doses of AH, the peak CL leveled off (6 to 8 HU_{50}/ml) and began to fall (10 to 20 HU_{50}/ml).

Effect of AH on leukocyte phagocytosis. To determine the effect of AH on leukocyte phagocytosis, HPL were treated with 2 HU_{50} of AH per ml or with an equal volume of buffer. Approximately 80% of HPL treated with 2 HU_{50} of AH per ml remained viable as determined by trypan blue uptake. After 30 min of incubation at 37°C, the cells were washed, harvested by cen-

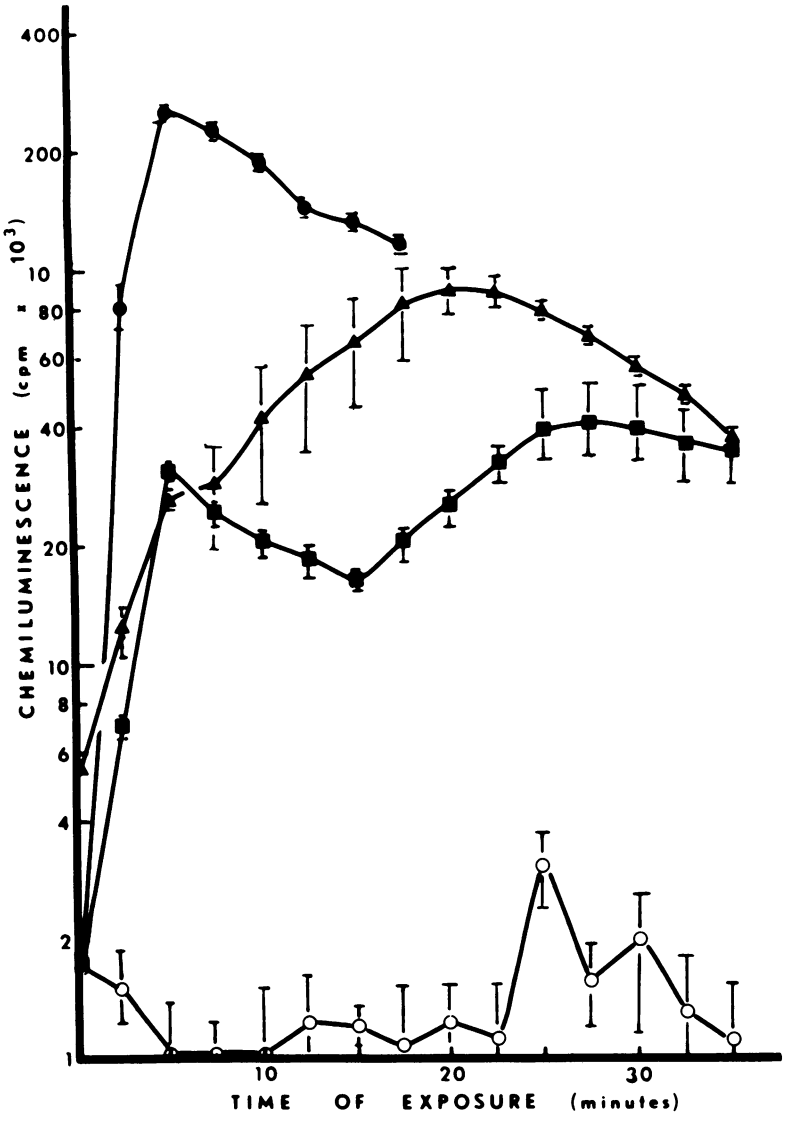

FIG. 1. CL of leukocytes exposed to low doses of AH. A total of 10^6 leukocytes per ml were exposed to: ■, 1 HU_{50} of AH per ml; ▲, 2 HU_{50} of AH per ml; ●, 100 μg of concanavalin A per ml; ○, buffer. Brackets represent the range of activity in repeated experiments.

trifugation, and adjusted to the same number of viable cells per unit volume in BSA-HBA. These treated HPL (5×10^5 cells per ml) were added to vials, and the background CL for the resting state was measured for 10 min after which 5×10^7 CFU of opsonized *S. epidermidis* per ml were added. The CL was then measured over the next 60 min (Fig. 4). The levels of CL in AH treated HPL were threefold less than those in buffer-treated cells. This threefold difference was established early in the incubation period (2 to 5 min after the addition of *S. epidermidis*) and was maintained throughout. Bacteria alone did not exhibit CL to any significant degree (2 to 3 logs less than bacteria plus HPL).

To obtain more evidence that AH impairs phagocytosis, the survival and attachment of bacteria exposed to AH- or buffer-treated HPL were measured by direct plate counts. Opsonized *S. epidermidis* was added to AH- or buffer-treated HPL to give a 2:1 bacterium-to-leukocyte ratio. A control vial contained bacteria alone. The mixtures were incubated at 37°C for 1 h, at which time samples were removed, and

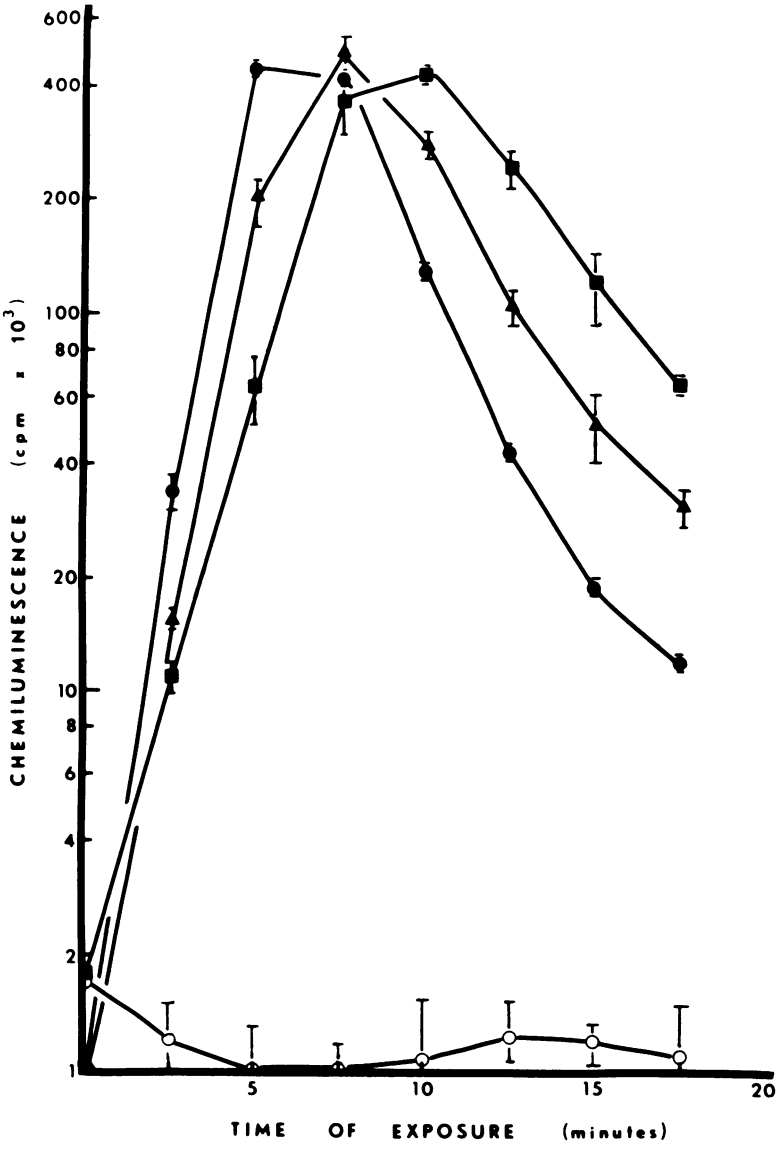

FIG. 2. CL of leukocytes exposed to high doses of AH. A total of 10^6 leukocytes per ml were exposed to: ■, 6 HU_{50} of AH per ml; ▲, 8 HU_{50} of AH per ml; ●, 10 HU_{50} of AH per ml; ○, buffer. Brackets represent the range of activity in repeated experiments.

TABLE 1. Effect of low doses of AH on leukocyte phagocytosis as measured by the attachment and survival of *S. epidermidis*

Treatment of leukocytes[a]	% Attached			% Survival		
	Mean	Range[b]	% Control	Mean	Range	% Control
2 HU_{50} of AH per ml	13	5–21	26	91	87–95	60
Equal volume of buffer	50	37–63	100	55	53–57	100

[a] Leukocytes were pretreated with each reagent as described in the text.

[b] Values were adjusted for variations in the control vial which contained bacteria without leukocytes. Data are from two separate experiments.

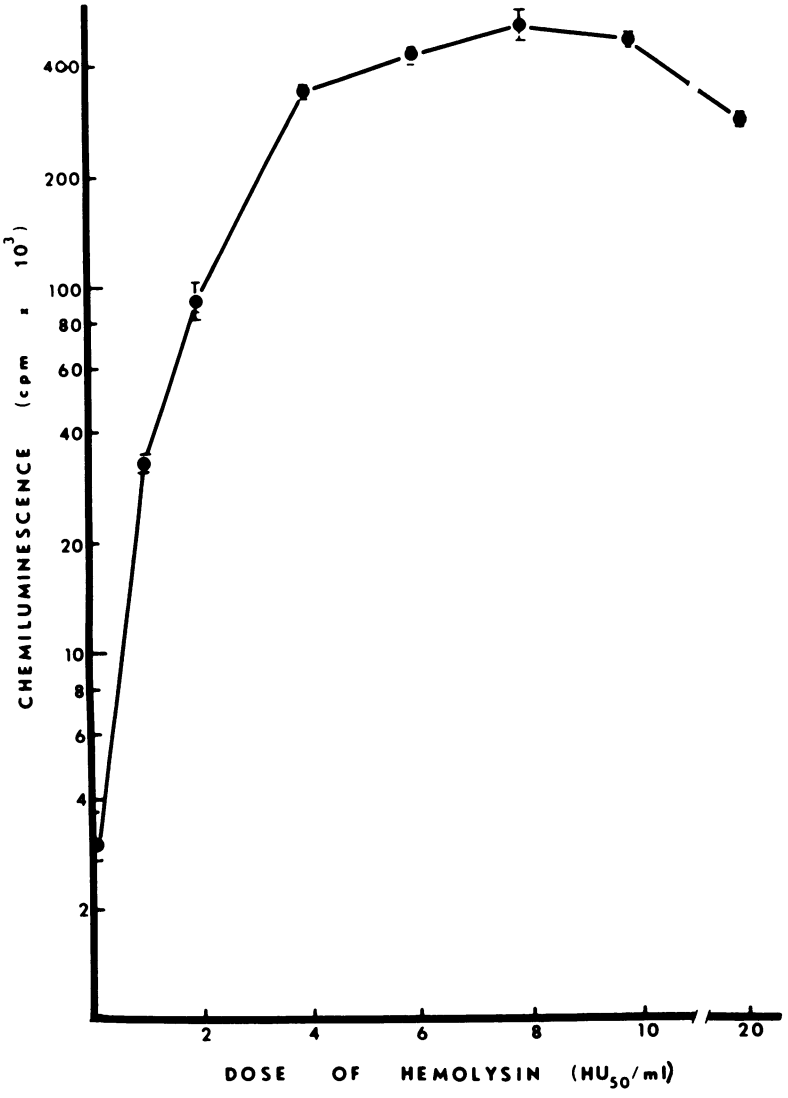

FIG. 3. Dose response of the CL of 10^6 leukocytes per ml exposed to various doses of AH (●). The peak CL was plotted for each dose of AH. Brackets represent the range of activity in repeated experiments.

CFU were measured by plate counts. The results of two separate experiments are shown in Table 1. The attachment of bacteria to AH-treated HPL was reduced to 26% of that obtained with buffer-treated cells, whereas the level of killing of bacteria was reduced to 60% of that of the control cells. Similar results were obtained in experiments in which the mixtures were incubated for 2 h except that the number of bacteria attached and the number of bacteria killed increased in both systems. The impairment of attachment and killing by AH-treated cells was still evident.

Finally, the AH effect on HPL phagocytosis was measured spectrophotometrically by ingestion of oil red O-lipopolysaccharide particles. Particles prepared as described above were added to AH- or buffer-treated HPL (10^6 to 10^7 viable cells per ml). After 5 min of incubation at 37°C, the cells were washed and extracted as described above, and the optical density at 525 nm of the extracts was measured. The results of two separate experiments are shown in Table 2. The ingestion rate of AH-treated cells was reduced to approximately 70% of that of buffer-treated cells.

Effect of AH on HPL chemotaxis. To determine the effect of AH on HPL chemotaxis, AH- or

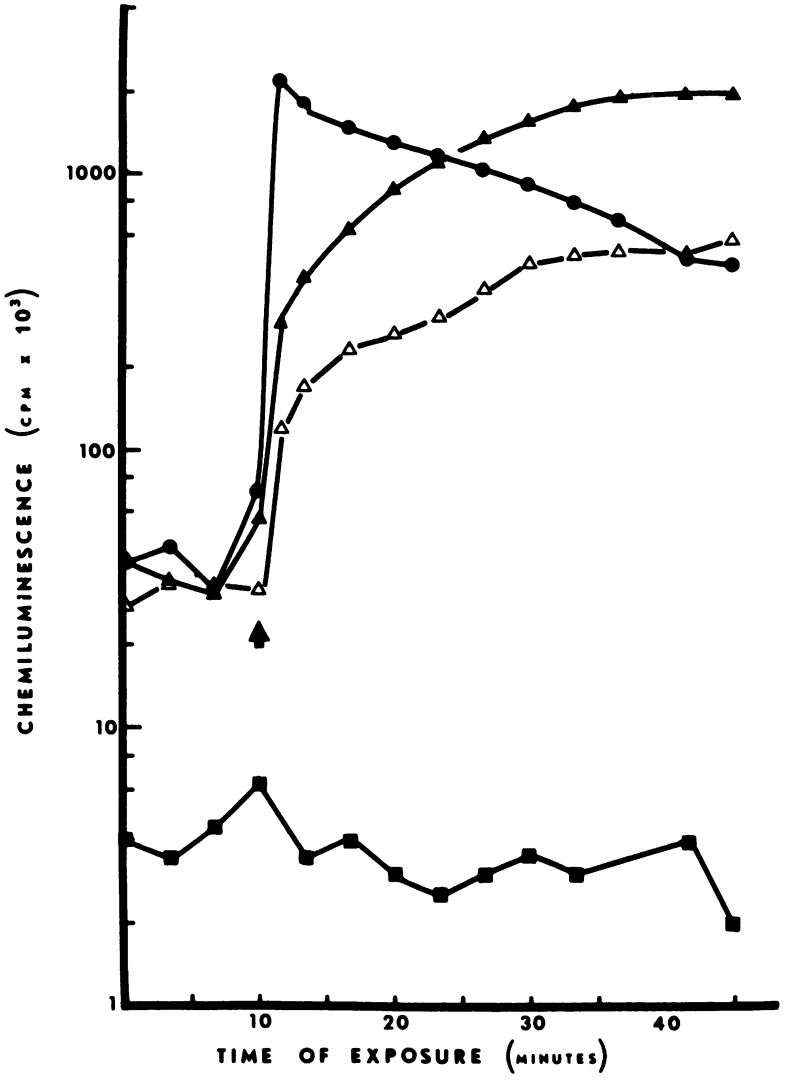

FIG. 4. Effect of low doses of AH on leukocyte phagocytosis as measured by CL. Symbols: △, 5×10^5 leukocytes per ml pretreated with 2 HU_{50} of AH per ml then exposed to 5×10^7 CFU of opsonized *S. epidermidis* per ml; ▲, 5×10^5 leukocytes per ml pretreated with an equal volume of buffer and then exposed to 5×10^7 CFU of opsonized bacteria per ml; ●, 5×10^5 leukocytes per ml pretreated with buffer and then exposed to 100 μg of conconavalin A per ml; ■, 5×10^7 CFU of opsonized bacteria per ml without added leukocytes. Arrow indicates the time of addition of the stimulant (opsonized bacteria or conconavalin A).

buffered-treated cells (3×10^6) were added to the upper half of a modified Boyden chamber as described above (Table 3). Chemotaxis by AH-treated cells was reduced 2.6-fold when compared with that of buffer-treated cells.

DISCUSSION

Leukocyte metabolic activation as measured by CL was stimulated by AH. The CL response of HPL exposed to AH was time and dose dependent. Cells exposed to as little as 1 HU_{50}/ml exhibited a biphasic time course of CL. This biphasic response is unexplained at present, but we suggest that early CL may reflect the initial binding of AH to a small percentage of the cells that subsequently undergo lysis (6), with a consequent drop in CL. The release of intracellular enzymes and other constituents from these cells may then stimulate the remaining cells, causing a second peak of CL. At higher doses of AH (2 HU_{50}/ml or greater), the time course kinetics were not biphasic but increased more sharply and to a higher degree and fell off more rapidly. These results may suggest that at higher hemoly-

TABLE 2. Effect of low doses of AH on leukocyte phagocytosis as measured by ingestion of oil red O-lipopolysaccharide particles

Treatment of leukocytes[a]	Ingestion rate[b]		% Control
	Mean	Range	
2 HU_{50} of AH per ml	79	74–84[c]	70.8
Equal volume of buffer	112	96–127[c]	100

[a] Leukocytes were pretreated with each reagent as described in the text.

[b] Ingestion rate is expressed as micrograms of diisodecylphthalate per 10^7 cells per minute.

[c] Range of two separate experiments.

sin/cell ratios a majority of the cells were probably immediately affected by AH, resulting in a higher CL than at lower doses. When these cells lyse (6), there are few, if any, remaining cells to be affected by leukocyte intracellular constituents. No lag phase was evident in the CL response of HPL exposed to AH at any dose. This is in contrast to the data of Andersen and Duncan (2), who indicate a 1- to 3-min lag phase in the CL response of human neutrophils exposed to streptolysin O (SLO).

A proportional relationship was seen between the concentration of AH and the peak CL over a concentration range of 0 to 4 HU_{50}/ml. At high doses (6 to 8 HU_{50}/ml), CL leveled off. Even higher doses (10 and 20 HU_{50}/ml) gave a lower peak CL response. These data may be explained by the increased and more rapid leukocyte lysis at these high AH concentrations (6). Andersen and Duncan (2) reported similar data for the effect of SLO on human neutrophils. They found that CL increases as the dose of SLO is increased from 0 to 18 HU_{50}/ml, with 3 HU_{50}/ml being the minimum amount required to stimulate CL. At higher doses of SLO, CL is decreased due to increased neutrophil lysis.

The mechanism whereby treatment with AH results in CL is unknown. One can speculate that perturbation of the leukocyte cell membrane by AH may itself be sufficient to activate the enzymes needed for CL. Alternatively, membrane permeability may be altered by treatment with AH, thereby resulting in an influx of ions which may activate leukocyte metabolism. Andersen and Duncan (2) demonstrated a calcium ion dependence for SLO-induced CL in human polymorphonuclear leukocytes.

AH-treated HPL exposed to opsonized *S. epidermidis* exhibited a threefold-lower CL response than did buffer-treated HPL. The legitimacy of using CL as a measure of leukocyte phagocytosis is the subject of some controversy. Karnovsky (15) described the respiratory burst phenomenon that results from activation during phagocytosis. Allen et al. (1) proposed that the bactericidal activity of leukocytes is due to the production of a highly reactive O_2 intermediate during the respiratory burst. They reasoned that leukocytes should exhibit a CL response with the onset of phagocytosis since CL is dependent upon these O_2 intermediates. These workers found that leukocytes exposed to opsonized bacteria exhibit CL, whereas normal cells do not. In addition, Nelson et al. (21) found that phagocytizing human monocytes also exhibit a CL response. Mangan and Snyder (18) showed that HPL exhibit CL when exposed to piliated *E. coli* and that this CL corresponds with bactericidal activity (17). In contrast, Welch (36) found that there is no complete correlation between peak, slope, or curve area of the CL response and bacterial susceptibility to phagocytosis with four serotypes of *E. coli*. However, CL and susceptibility to phagocytosis can be correlated if the bacteria are opsonized.

Since CL is an indirect method of measuring leukocyte phagocytosis, the effect of AH was measured directly by plate counts. Pretreatment of HPL with low doses of AH reduced killing of opsonized *S. epidermidis* to 60% of that obtained with buffer-treated cells. The effect on phagocytosis is not clear, but it may be at the level of bacterial attachment to the leukocytes since attachment was reduced in AH-treated cells to 26% of that of control cells. The decreased attachment and phagocytosis by both plate counts and CL suggest that some receptors on the leukocyte cell membrane may be destroyed or modified by AH.

Phagocytosis was also measured spectrophotometrically by the method of Stossel (30), which involves the ingestion of oil red O-lipopolysaccharide particles. Phagocytosis of these particles by AH-treated HPL was reduced to 70% of that of control cells. Differences in the degree of reduction in the phagocytosis assays

TABLE 3. Effect of low doses of AH on leukocyte chemotaxis

Treatment of leukocytes[a]	Attractant	No. of migrating leukocytes[b]	% Control
2 HU_{50} of AH per ml	ZAS	149 ± 25	38.7
	0.1% BSA–HBS	4 ± 2	50.0
Equal volume of buffer	ZAS	385 ± 108	100
	0.1% BSA–HBS	8 ± 3	100

[a] Leukocytes were pretreated with each reagent as described in the text.

[b] Represents the mean number ± standard deviation of leukocytes in five high-power fields migrating through a filter. The data represent an average of three experiments.

(plate counts, oil red O uptake, and CL) are probably due to their various sensitivities. Ofek and associates (23) reported similar data for the effect of SLO and streptolysin S on phagocytosis of killed streptococci by mouse peritoneal macrophages.

Chemotaxis of leukocytes was also affected by AH. Only one-third of the leukocytes pretreated with low doses of AH were able to migrate toward ZAS as compared with control cells. Others (3, 32) reported similar data for SLO. Treatment of human neutrophils with as little as 0.12 HU_{50} of SLO per ml reduced chemotaxis to 8 to 46% of that of control cells. Treatment with 1 HU_{50}/ml reduced chemotaxis to 0 to 13% of that of control cells (3).

The mechanisms for the effects of SLO and AH on leukocyte phagocytosis and chemotaxis are unknown. However, it is possible that these two effects are interrelated. Andersen and Van Epps (3) have suggested that the effect of SLO on neutrophil chemotaxis may be due to the disruption of the cellular mechanism responsible for cell mobility. This may also be true for AH. Alternatively, AH may affect the surface receptor for the opsonized chemotactic stimuli. Chenoweth and Hugli (8) clearly demonstrated the existence of a specific C5a receptor on human leukocytes that is involved in the process of leukocyte chemotaxis toward a gradient of opsonized particles. Modification of this receptor by the action of AH on the cell membrane may suppress the ability of leukocytes to undergo chemotaxis. If the ability of leukocytes to undergo chemotaxis is impaired, their ability to phagocytize opsonized particles will also be impaired since the cells cannot efficiently migrate toward them. This hypothesis is partially supported by the fact that attachment of opsonized bacteria to AH-treated leukocytes was reduced to 26% of that of control cells.

In summary, the above data suggest a possible role for AH in pathogenesis. It is apparent that AH adversely affects leukocyte viability (6) and function in vitro. Whether these activities occur in vivo is unknown. If we assume that these activities do occur in vivo, a likely scenario may be that proposed by Andersen and Duncan (2) for SLO. At low concentrations of AH such as those found at greater distances from the focus of infection, leukocytes (primarily neutrophils) may be impeded in their chemotactic movement toward invading *E. coli* cells. As the AH concentration slowly increases at sites closer to the focus of infection, the leukocytes may be prematurely activated, leading to reduced bactericidal capacity and to the release of intermediates which can in turn damage host tissues and cause a harmful inflammatory reaction. Closer to the focus of infection, where AH concentrations would be higher, leukocyte lysis may occur, and any cells surviving would probably have a greatly reduced capacity to phagocytize bacteria. All these events should increase *E. coli* survival.

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