

## Defensins Impair Phagocytic Killing by Neutrophils in Biomaterial-Related Infection

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**The implantation of foreign material carries a risk of infection which frequently is resistant to all treatment short of removing the implant. We have previously shown that these materials activate neutrophils by contact, leading to production of oxygen free radicals accompanied by release of granule products. Such activation further results in depletion of local host defenses, including the capacity of biomaterial-activated neutrophils to kill bacteria. Among the granule products released from neutrophils are small cationic antibacterial peptides (human neutrophil peptides [HNP]) known as defensins. Here we tested the hypothesis that defensins, released from activated neutrophils onto the surface of biomaterials, might play a role in the deactivation of subsequent neutrophil populations. Incubation of neutrophils with purified HNP resulted in a dose-related impairment of stimulus-induced oxygen radical production and of phagocytic killing. Furthermore, fresh neutrophils added to biomaterial-associated neutrophils exhibited impaired phagocytic killing. This impairment could be abrogated by antibody to HNP but not by an irrelevant antibody. Taken together, these observations support the idea that neutrophils activated at a material surface can create, by means of HNP release, an environment hostile to their microbicidal function and that of their infiltrating brethren.**

Implantable biomaterials, virtually indispensable in medical practice (16, 22, 29) and with an excellent overall success rate, have continued to be infection prone, with enormous social and economic consequences (13, 14, 23, 25, 26, 40, 66, 76). These infections tend to be persistent and refractory to antibiotics and often require removal of the implant to clear the infection (14, 22, 40). In this sense, they resemble abscesses, which require surgical drainage in order to be eradicated.

The pathogenic mechanisms of this refractoriness to treatment, however, have remained obscure. Contributory factors include bacterial virulence properties, such as slime production (4, 10, 23, 31, 56, 60), as well as properties of specific biomaterials which increase microbial adherence or alter inflammatory changes (1, 2, 7, 9, 12, 23, 24, 27, 30, 51, 53, 58, 70, 71, 77, 78) and impaired phagocytic host defenses even in the absence of microbial colonization (34–36, 57, 59, 64, 78, 79).

Neutrophils rapidly become associated with any implanted biomaterial *in vivo* and, under conditions permissive of normal function, should be capable of phagocytic host defense. Previous work by others (2, 15, 21, 27, 30, 39, 41, 52, 53, 57–59, 64, 77–79) and in our laboratory (34–36), however, has shown that biomaterial-associated neutrophils become prematurely activated by contact with the materials themselves and shortly thereafter lose the capacity to become activated in response to normal subsequent stimuli. More importantly, these biomaterial-associated neutrophils appear to actually induce a dysfunctional impaired activation of incoming fresh neutrophils (36, 78, 79).

Normally, neutrophils effect phagocytic host defense by ingesting and killing invading microorganisms (3, 54, 69). The killing of microbes occurs as a consequence of reactive oxygen intermediates (ROI) formed during the process of phagocytosis together with bioactive constituents from granules which

discharge into the phagocyte vacuole (5, 62, 63, 72, 73). These constituents include myeloperoxidase, which, when combined with H<sub>2</sub>O<sub>2</sub> and chloride, produces hypochlorous acid within the phagocytic vacuole, as well as small highly cationic peptides (human neutrophil peptides [HNP]), also known as defensins. The defensins HNP1, -2, and -3 comprise 5% of a neutrophil's total protein (19, 44, 46). HNP contain 29 to 35 amino acid residues, and because of their charge and their tendency to form multimeric subunits, they insinuate themselves into microbial cell membranes (gram-positive and gram-negative bacteria, fungi, and viruses) (11, 45, 47, 61), produce voltage-sensitive channels, and thereby permeabilize the membrane (33). HNP also are known to damage eukaryotic cells, such as tumor cells (48, 65) and human neutrophils (75), and in a cell-free system they have been shown to inhibit NADPH oxidase (67). This cytotoxicity depends in part on the lipid composition of the target cell membrane (28) and requires metabolic activity in the target cells (49). In addition, hydrogen peroxide acts synergistically with HNP to induce cytotoxicity (50). Along with permeabilization, DNA strand breaks in some target cells have been reported (20, 49), and while the mechanism has not been determined, a direct interaction between defensin and DNA is suspected.

Because HNP may be toxic to polymorphonuclear leukocytes (PMN) (67, 75), and because material-activated neutrophils could be expected to release HNP from their granules (5, 62, 63, 72, 73), we proposed that the dysregulated functioning of incoming fresh neutrophils (36) might be induced by these released HNP. To further explore this idea in the context of biomaterial-induced changes, the effect of purified HNP on phagocytic killing by neutrophils was evaluated and demonstrated a dose-related cytotoxicity of HNP1 and HNP2 ameliorated by antibody to HNP. These data support the idea that released HNP contribute to an environment hostile to host defense at the biomaterial surface.

### MATERIALS AND METHODS

**Cells and reagents.** Blood was obtained from healthy human donors. Neutrophils were isolated by density gradient centrifugation according to the method of

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Boyum (6), as previously described (37, 43), and suspended in modified Krebs Ringer phosphate buffer, pH 7.4 (16 mM  $\text{Na}_2\text{HPO}_4$ , 123 mM NaCl, 0.5 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgSO}_4$ , 5 mM KCl, and 4.4 mM glucose) (KRPG) (35). In some studies, the KRPG included 10% autologous plasma. Each neutrophil donor provided cells for a single day's experiments. Cell numbers were determined with Coulter (Hiאהא, Fla.) electronic instruments, and the cells were suspended at a concentration of  $2 \times 10^6/\text{ml}$ . The preparations were evaluated with DiffQuick-stained cytospin preparations and were uniformly >95% neutrophils. Viability was determined by trypan blue dye exclusion and was uniformly >95%. Stock solutions of formyl methionyl leucyl phenylalanine (fMLP), phorbol myristate acetate (PMA), and opsonized zymosan (OZ) were prepared as previously described (34). Purified human neutrophil defensins HNP1 and HNP2 were obtained from Sigma Chemical Co., St. Louis, Mo., and stored at  $-20^\circ\text{C}$  until needed. At that time, the HNP were suspended in phosphate-buffered saline (PBS) at a concentration of 25  $\mu\text{g}/\text{ml}$  and were diluted in PBS so that the PMN were incubated with 10 to 35  $\mu\text{g}$  of HNP/ml. Monoclonal antibody to HNP (D1-1 immunoglobulin G1, a neutralizing antibody) was a generous gift of T. Ganz, University of California—Los Angeles School of Medicine, Los Angeles. The stock solution of 10,000  $\mu\text{g}/\text{ml}$  (10 mg/ml) was diluted in PBS to the desired concentration just before use. Antibody to VCAM-1 (an irrelevant immunoglobulin G1 control antibody) was a generous gift from T. Carlos, University of Pittsburgh. It was in a stock solution of 3,600  $\mu\text{g}/\text{ml}$  (3.6 mg/ml) and was appropriately diluted in PBS just before use. Unless specified, all other materials were reagent grade and were obtained from Sigma Chemical Co.

**Superoxide release.** Human neutrophils ( $10^6$ ) were preincubated with purified human neutrophil defensins and then were placed into triplicate wells of 24-well plates in KRPG. Cytochrome *c* was added with and without fMLP, PMA, OZ, or *Staphylococcus aureus*, and the plates were incubated at  $37^\circ\text{C}$  for 30 min. Superoxide dismutase was present in a fourth well. The superoxide dismutase-inhibitable cytochrome *c* reduction was determined after 30 min, at which time the plates were placed in melting ice and centrifuged and the supernatants were transferred to 96-well plates. The data are expressed as nanomoles of  $\text{O}_2^-/10^6$  cells.

**Microbe killing.** The effects of biomaterial association, of antibody to HNP, and of HNP1 and HNP2 on microbe killing were determined by clonal-culture techniques as previously described, using *S. aureus* D2C as the target organism (38).

The effect of HNP1 and HNP2 on the ability of PMN to kill staphylococci was determined by preincubating PMN in KRPG ( $10^6/\text{ml}$ ) in 12- by 75-mm test tubes with various concentrations of HNP1 and HNP2 at  $37^\circ\text{C}$  for 1 h. The 1-h incubation was based on published data demonstrating that toxicity for mammalian eukaryotic cells requires 1 h for completion and irreversibility (44, 49). The PMN were then centrifuged, washed once with KRPG, and resuspended in KRPG plus 10% plasma;  $5 \times 10^5$  cells were put in 25-ml Erlenmeyer flasks and placed in a rotary shaking water bath. After 10 min, an inoculum of *S. aureus* was added (*Staphylococcus*/neutrophil ratio, 10:1). The samples were swirled to mix them, and a 10- $\mu\text{l}$  sample was removed immediately after the addition of bacteria (the time zero sample). Samples (10  $\mu\text{l}$ ) were removed at 2 and 24 h, and the numbers of viable staphylococci were determined.

The effect of biomaterial-associated PMN on the microbicidal activity of a fresh inoculation of PMN was determined by first incubating the PMN in KRPG in 24-well culture dishes for 1 h at  $37^\circ\text{C}$  and then adding a similar number of PMN and continuing the incubation for 1 h more. The PMN were removed from the wells after the addition of EDTA to reverse the adhesion phenomenon and then centrifuged and washed prior to being resuspended in KRPG and placed in the wells of a clean 24-well culture dish. For control conditions, PMN were stored at  $37^\circ\text{C}$  for 1 h in a test tube and then added to complete buffer containing 10% plasma. *S. aureus* was added 10 min later, and bacterial colony counts were made at the time of *S. aureus* addition as well as after 2 and 24 h.

To determine if incubating PMN with antibody to HNP (D1-1) resulted in improved staphylocidal activity of fresh PMN added to biomaterial-associated PMN, various concentrations of the antibody to HNP (2 to 200  $\mu\text{g}/\text{ml}$ ) or to VCAM at 200  $\mu\text{g}/\text{ml}$  were placed in the wells of 24-well plates just prior to the addition of the initial inoculum of PMN. The PMN were in contact with the wells, with and without the presence of antibody, for 1 h at  $37^\circ\text{C}$ . In some experiments, 50 and 80% plasma were added instead of antibody. A second inoculation of PMN was added, as described above, and incubation continued for 1 h more at  $37^\circ\text{C}$ . These PMN, washed and resuspended in KRPG with 10% plasma, were transferred to a fresh well; staphylococci were then added, and incubation continued for 24 h as described above.

**Defensin assay.** The amount of available defensins in PMN before and after exposure to polystyrene was determined by Panyutich's method. Briefly, this is a "sandwich-type" enzyme immunoassay with a monoclonal antibody for capture and a biotinylated monoclonal antibody for detection. Cetyltrimethyl ammonium bromide was used to counteract nonspecific binding of HNP to surfaces. The assay detects HNP with a range of 0.5 to 16 ng/ml.

**Statistical evaluation.** Calculations of statistical significance were done with Student's *t* test or the paired *t* test. Significance was defined as a *P* value of  $\leq 0.05$ .

## RESULTS

Figure 1 illustrates the effect on bacterial viability of adding a secondary inoculum of fresh neutrophils to polystyrene wells

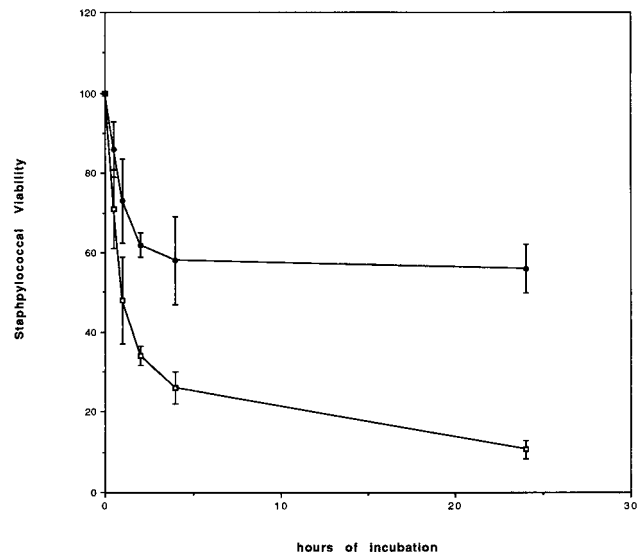


FIG. 1. Effect of polystyrene preincubation on staphylococidal activity. The percent viable staphylococci during 24 h of incubation with neutrophils is shown. Control neutrophils were inoculated with *S. aureus* at the same time the cells were placed in the polystyrene wells (□). The decrease in the number of viable staphylococci occurred rapidly during the first 2 h of incubation and more slowly after that. These cells killed  $65\% \pm 3\%$  of the inoculum after 2 h and  $88\% \pm 2\%$  of the inoculum after 24 h. The figure also shows the effect on the staphylocidal behavior of fresh neutrophils added to neutrophils that had been preincubated for 1 h in polystyrene wells (●). These cells killed only  $38\% \pm 4\%$  of the inoculum at 2 h and  $41\% \pm 8\%$  of the inoculum at 24 h ( $P \leq 0.001$  compared to the control at both of these times). The error bars indicate standard errors.

containing the primary inoculum of neutrophils which had been on the polystyrene surface for 1 h. These neutrophils killed  $38\% \pm 4\%$  of the *S. aureus* organisms in 2 h and killed  $41\% \pm 8\%$  of the organisms by 24 h. In contrast, when the *S. aureus* organisms were added within 10 min of the primary inoculation of PMN onto the polystyrene,  $65\% \pm 3\%$  of the *S. aureus* organisms were killed in 2 h and  $88\% \pm 2\%$  were killed by 24 h ( $P \leq 0.001$ ). These data indicate that the association of the first inoculum with polystyrene for 1 h down-regulated microbe killing by the secondary inoculum.

One likely cause for this effect was the release of granule products, such as HNP, with documented (11, 19, 20, 44–50, 61, 65) toxicity against both eukaryotic and prokaryotic cells. We therefore examined the supernatants of polystyrene-associated PMN (after 1 h of association) for the presence of HNP. Less than 1,000 ng was detected, too low a concentration to explain our results based on published data. However, since strongly cationic HNP readily adhere to negatively charged surfaces such as plastic (55) and to cells (17), the poor recoverability of the HNP may have represented an artifact. Residual intracellular HNP from control PMN and from PMN after polystyrene association was then measured and is shown in Fig. 2. In this study, a marked reduction in the detectable HNP content of biomaterial-associated cells was seen, from  $3,616 \pm 1,098$  ng/ml/ $10^6$  PMN extractable in control cells to  $1,313 \pm 183$  ng/ml extractable 30 min after polystyrene exposure and  $886 \pm 277$  ng/ml extractable at 2 h. We also evaluated defensin content in cells exposed to woven Dacron and Silastic, finding  $453 \pm 230$  and  $1,275 \pm 215$  ng/ml, respectively. These results are consistent with the idea that PMN associated with biomaterials discharge their granular HNP and that the released HNP become associated with or inactivated by the polystyrene. To test this hypothesis, we added purified HNP1 and HNP2 to polystyrene plates in the absence of cells. The supernatants were analyzed for residual

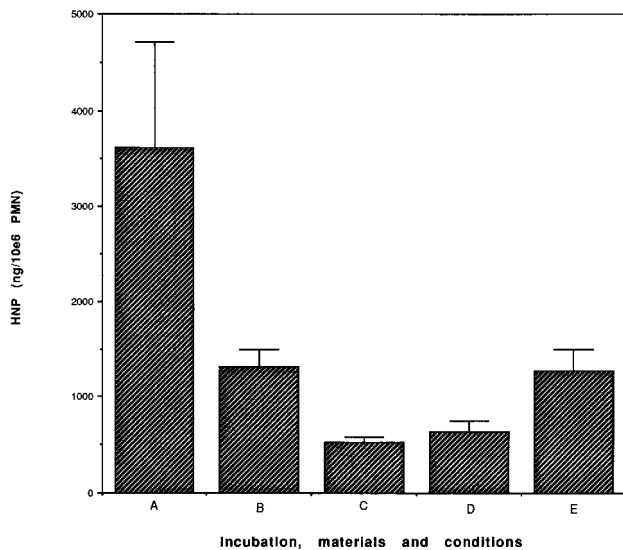


FIG. 2. Extractable defensin of control neutrophils (A), cells which had been incubated on polystyrene for 30 min (B) and for 2 h (C), and neutrophils incubated for 2 h on woven Dacron (D) or silastic (E). Exposure to all three materials was associated with significantly reduced defensin content ( $P \leq 0.01$  under all conditions). The error bars indicate standard errors.

HNP. Detectable HNP disappeared within 15 min, with only 2% remaining in the supernatant. These results are consistent with those of others (55), showing rapid association of HNP with plastic.

To determine whether HNP might impair PMN staphylocidal activity, commercially available purified HNP were incubated with PMN for 1 h at 37°C. The ability of these incubated and washed PMN to kill *S. aureus* is shown in Fig. 3. Figure 3A shows that HNP1, at a concentration of 35  $\mu\text{g/ml}$ , impaired the staphylocidal activity of PMN such that only 31%  $\pm$  13% and 37%  $\pm$  11% of the inoculum was killed after 2 and 24 h of incubation, respectively ( $P \leq 0.01$  compared with the control or with HNP1 at 10  $\mu\text{g/ml}$  and  $P \leq 0.03$  compared with HNP1 at 25  $\mu\text{g/ml}$ ). Incubation of PMN with HNP1 at 25  $\mu\text{g/ml}$  also compromised staphylocidal activity, but the inhibition was more variable. Figure 3B shows that HNP2 is a more potent inhibitor of PMN staphylocidal activity. The use of 25  $\mu\text{g/ml}$  almost completely abrogated staphylocidal activity after 2 and 24 h of incubation ( $P \leq 0.01$  compared to the control and to HNP2 at 10  $\mu\text{g/ml}$ ). As little as 15  $\mu\text{g}$  of HNP2/ml also significantly impaired staphylocidal activity ( $P \leq 0.01$  at 2 and 24 h compared to the control and  $P \leq 0.05$  at 2 and 24 h compared to HNP2 at 10  $\mu\text{g/ml}$ ). HNP2, therefore, was far more toxic to PMN than HNP1 was. In addition, Fig. 4 shows that HNP1, at 35  $\mu\text{g/ml}$ , significantly impaired the ability of neutrophils to produce superoxide following stimulation with any of the inducing agents. Parallel experiments were performed with HNP2, which demonstrated complete abrogation of superoxide release at 20  $\mu\text{g/ml}$  (data not shown). These cytotoxic correlations of HNP and the incubation conditions are consistent with the known conditions for cytotoxicity of HNP for eukaryotic cells (18, 32). Despite these inhibitory effects of HNP on microbe killing and  $\text{O}_2^-$  release, HNP had no effect on PMN viability as determined by trypan blue dye exclusion during the first 5 h following PMN incubation with 35  $\mu\text{g}$  of HNP1/ml, and viability was 73% after 24 h.

To further evaluate the likelihood that HNP released from polystyrene-associated PMN can act to disable PMN (Fig. 1), antibody to HNP was added to the first inoculum of PMN

placed in the polystyrene wells. Figure 5 shows that this antibody reversed the cytotoxic effect at both time points ( $P \leq 0.05$  at 2 h;  $P = 0.0001$  at 24 h) when killing in the presence and absence of 200  $\mu\text{g}$  of specific anti-HNP antibody/ml were compared. Lower concentrations of antibody (2 to 50  $\mu\text{g/ml}$ ) were without effect (data not shown), as was the use of 200  $\mu\text{g}$  of an irrelevant antibody (antibody to VCAM)/ml at 2 and 24 h.

## DISCUSSION

An implanted biomaterial foreign body has paradoxical effects; it induces the accumulation of phagocytes and at the same time can become the nidus of intractable bacterial infec-

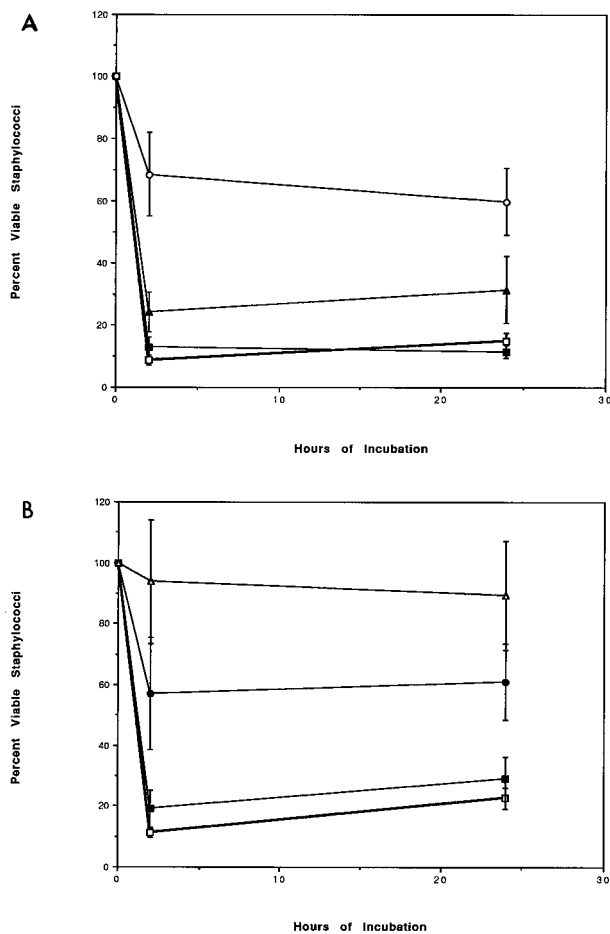


FIG. 3. (A) Effect of HNP1 on staphylocidal activity. The effect of preincubating neutrophils for 1 h with several concentrations of HNP1 (or buffer control) is shown. The cells were washed and resuspended in KRPG with 10% plasma and then inoculated with staphylococci. The control cells (□) killed 91%  $\pm$  1% of the inoculum after 2 h and 88%  $\pm$  2% of the inoculum after 24 h. HNP1 at 10  $\mu\text{g/ml}$  (■) did not affect staphylocidal activity. HNP1 at 25  $\mu\text{g/ml}$  (▲) reduced staphylocidal activity to 75%  $\pm$  7% killed at 24 h ( $P \leq 0.03$  compared to the control or HNP1 at 10  $\mu\text{g/ml}$ ). HNP1 at 35  $\mu\text{g/ml}$  (○) reduced staphylocidal activity to 31%  $\pm$  13% killed at 2 h and 37%  $\pm$  13% killed at 24 h ( $P \leq 0.01$  compared to the control). (B) Effect of HNP2 on staphylocidal activity. The effect of HNP2 on the staphylocidal activity of human PMN is more potent than the effect of HNP1 shown in panel A. Although HNP2 at 10  $\mu\text{g/ml}$  (■) did not significantly affect killing, HNP2 at 15  $\mu\text{g/ml}$  (●) partially inhibited staphylocidal activity: 41%  $\pm$  20% of the inoculum was killed at 2 h, and 40%  $\pm$  8% was killed at 24 h compared to 89%  $\pm$  5% and 85%  $\pm$  2% killed at 2 and 24 h by control cells (□) ( $P \leq 0.01$  at 2 and 24 h). HNP2 at 25  $\mu\text{g/ml}$  (△) almost completely abrogated staphylocidal activity: 5%  $\pm$  15% killed at 2 h and 8%  $\pm$  20% killed at 24 h ( $P \leq 0.01$  at 2 and 24 h compared to the control;  $P \leq 0.05$  at 2 and 24 h compared to HNP2 at 10  $\mu\text{g/ml}$ ).



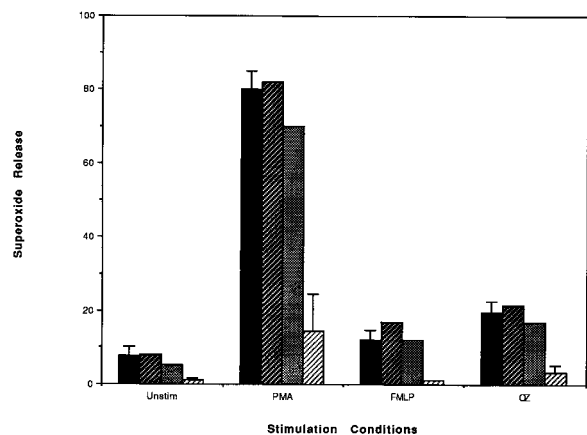


FIG. 4. Effect of HNP1 on superoxide release. Incubation of neutrophils with 35  $\mu\text{g}$  of HNP1/ml (▨) significantly reduced the superoxide release of unstimulated (unstim) cells and of cells stimulated with PMA, FMLP, and OZ ( $P \leq 0.01$ ) compared to unexposed (control) cells (■). Incubation with 17.5 (▨) and 25 (▩)  $\mu\text{g}$  of HNP1/ml had little effect (mean of two experiments).

tion. In fact, the greater the ability of a foreign body to induce a neutrophilic infiltrate, the greater its susceptibility to infection (12, 13, 30). Our previous work has indicated that PMN often become activated at the surfaces of biomaterials (34). The PMN can release chemoattractant substances, which would serve to attract fresh PMN to the surface (36). Such an event would be expected to potentiate host defenses but seems to have the opposite effect. We found that PMN, once in contact with biomaterials, rapidly lose their capacity to generate superoxide and are relatively impotent in their microbicidal activity (36). We subsequently showed that when fresh PMN were added to a surface upon which there had been a prior inoculum of PMN, the fresh PMN became inactive (36). These data, therefore, are consistent with the idea that the infectivity at biomaterial surfaces may be due at least in part to releasant-mediated autacoid and paracoid damage of the PMN themselves, rendering such wounds susceptible to even small inocula. Our present data support and extend our previous observation that fresh PMN, added to polystyrene-associated PMN and then removed and washed free of mediators, acquire a profoundly compromised staphylocidal activity. The data strongly suggest that a cytotoxic agent(s) is released when PMN contact polystyrene. These cytotoxic agents can further compromise the functioning of bystander PMN.

Neutrophils have considerable cytotoxic potential and play a role in many kinds of inflammatory reactions, ranging from autoimmune processes to reperfusion injury. This cytotoxic potential includes the metabolic products derived from the production of oxidative free radicals and the cytoactive granule constituents. The small cationic HNP, the defensins, comprise a potential subset of cytotoxic elements with well-established toxicity against prokaryotic and eukaryotic cells. The toxicity of HNP for bacteria is based on the ability of HNP to permeabilize the outer and inner membranes of susceptible bacteria (44) by the formation of ion channels (33) and by antagonizing intracellular signaling mediated by protein kinase C (8). These actions, however, are not limited to HNP, since Yeaman et al. demonstrated a similar, although not identical, antibacterial action of peptides from platelets (74). While several groups demonstrated that HNP were toxic to eukaryotic as well as to prokaryotic cells, the mechanism of injury to mammalian target cells is less well established. These actions have been reviewed by Kagan et al. (32), who indicated that there are three

interdependent actions, including (i) membrane binding due to electrostatic forces, followed by (ii) insertion into the membrane or internalization of the HNP (49). The third step (iii) includes DNA injury (20, 47), inhibition of NADPH oxidase (67, 68), and inhibition of protein kinase C (8). The initial binding becomes irreversible within 1 h of association, and although permeability to trypan blue dye was not observed in our study, the concentration of HNP may not have been high enough, since Lehrer indicated that HNP1 at 50  $\mu\text{g}/\text{ml}$  was needed to see this in PMN (personal communication). Mammalian cells have also been shown to require an energized target cell membrane, since metabolic inhibitors which do not effect initial binding do inhibit defensin-induced permeabilization. Yomogida et al. (75) specifically documented some of the results of HNP-induced cytotoxicity in PMN. These included diminished ROI formation, impaired phagocytosis (not due to impaired particle attachment), and increased adherence. Our work supports these findings and also demonstrates that the ability of PMN to kill bacteria is severely compromised.

Wright and Gallin (73) showed that PMN degranulate upon association with artificial materials in vitro. Klock and Bainton demonstrated that PMN associated with nylon wool initially degranulate but subsequently exhibit abnormal bactericidal activity (42). Our finding that PMN in polystyrene wells for 1 h lose intracellular defensin content and concurrently impair the function of subsequently added neutrophils strongly implicates the granular defensins in PMN dysfunction. We demonstrate in this paper that in the presence of antibody to HNP, but not of isotype-identical irrelevant antibody, the PMN placed in the polystyrene wells did not exert a cytotoxic effect on a fresh inoculum of PMN. The staphylococci added with these PMN were killed normally. These data support our hypothesis that HNP (among other possible releasants) from material-associated neutrophils play a significant role in the impairment of cell function at the material surface. Whether or not the defensins, which are apparently readily adsorbed onto polysty-

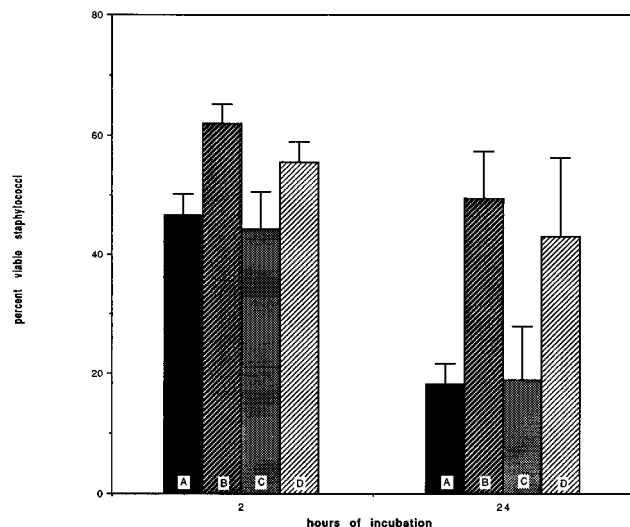


FIG. 5. Effect of antibody to HNP on polystyrene-associated inhibition of staphylocidal activity. Compared to control cells, in which staphylococci were added without a prior 1-h incubation of PMN on polystyrene (■), staphylocidal activity was impaired when fresh neutrophils were added to the polystyrene-exposed neutrophils (▨), but this is overcome by addition of monoclonal antibody specific for HNP (▩) but not by irrelevant antibody to VCAM (□) prior to the addition of fresh neutrophils. This effect was highly significant at both 2 and 24 h after the inoculation of neutrophils with staphylococci ( $P = 0.00008$ ).

rene, exert their anti-PMN function after adsorption is an important question that will require further study.

This work has not addressed the particular mechanisms by which HNP damage PMN in our system. We do not assert that defensin-mediated damage is the only route to neutrophil impairment on biomaterial surfaces, and Tal et al. (68) demonstrated that cationic proteins other than HNP may damage PMN oxidative activity. However, neutrophils also release proinflammatory mediators, such as oxidative free radicals, interleukin-8, and metabolites of arachidonic acid, including leukotrienes and prostaglandins, all of which could serve transiently to augment and potentially harm cell function. Our studies, however, clearly demonstrate that purified HNP incubated with PMN severely compromised phagocytic killing of staphylococci when used at a concentration of 25  $\mu\text{g/ml}$  for HNP2, a concentration determined by others to be toxic to eukaryotic cells, and at a concentration of 35  $\mu\text{g/ml}$  for HNP1. While most of our studies were performed after a 1-h incubation with HNP to ensure that the interaction of HNP with the cell membrane was irreversible, several studies also were performed after a much shorter (15-min) incubation. These studies also demonstrated profound inhibition of the PMN's killing of staphylococci (data not shown). These concentrations are much lower than HNP concentrations found in phagocytic vacuoles, so they could conceivably be achieved in the microenvironment of the degranulating cell. At lower concentrations which did not diminish phagocytic killing of staphylococci, superoxide production in response to a peptide stimulus (but not to a phagocytic stimulus) appeared to be very slightly greater than it was in the absence of HNP. At higher concentrations (sufficient to impair phagocytic killing), superoxide production was almost totally inhibited. These data are consistent with earlier studies in the 24-well culture plate model system, which demonstrated that the ROI production of secondary-inoculum PMN was diminished (36). The data are consistent with the finding that this biomaterial-associated impairment of ROI production could also be defensin related. It is even conceivable that defensins with intense cationic charge actually scavenge  $\text{O}_2^-$  and other antimicrobial radicals, such as  $\cdot\text{NO}$ . While events on one material should not be extrapolated to occurrences on other materials, preliminary studies have suggested that neutrophils placed on materials commonly used in medical practice, such as woven Dacron (a highly activating material) or Silastic (a less activating material but nevertheless associated with increased infectivity in vivo) also become depleted of defensins, with depletion on woven Dacron much greater than that on Silastic.

Taken together, these studies support our observations that neutrophil dysregulation at a material surface can contribute to microbial survival. It seems clear that released neutrophil products play some role in this dysregulation. These studies further show that one of these products is the granule constituent HNP, which not only plays an important role in host defense against microbial infection but can, under appropriate conditions, create an environment hostile to host defense processes.

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