Evidence for Reactive Nitrogen Intermediates in Killing of Staphylococci by Human Neutrophil Cytoplasts

A New Microbicidal Pathway for Polymorphonuclear Leukocytes

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Abstract

In anucleate, granule-poor, motile fragments from human blood neutrophils (cytokineplasts; CKP), the nitric oxide synthase inhibitor N^{ω}-monomethyl-L-arginine (NMMA) produced a modest decrease in uptake of staphylococci from supernatants ($P < 0.02$, $n = 7$), and a marked decrease in the killing of cytoplast-associated bacteria ($P < 0.001$, $n = 7$). After 60 min of incubation with bacteria, NMMA-treated cytoplasts had a mean of over 3.5 times as many live, CKP-associated staphylococci as did controls (51% of the inocula versus 14%), despite having taken up fewer. Effects on both uptake and killing were reversible by L-arginine but not by D-arginine. Results were the same with other granule-poor cytoplasts (U-cytoplasts, U-CYT), which, unlike CKP, retain activatable oxidase activity. Killing by intact PMN, including those from a patient with chronic granulomatous disease, was not inhibited by NMMA. Thus, the ability to discern effects of NMMA correlated with the paucity of granules, without regard to the presence or absence of activatable oxidase. We propose that the generation of reactive nitrogen intermediates serves as an additional microbial killing pathway in PMN, and that cytoplasts can be used to help delineate the spectrum of susceptible targets. (*J. Clin. Invest.* 1992. 90:631–636.) Key words: nitric oxide • cytokineplasts • U-cytoplasts

Introduction

It has rapidly become apparent in recent years that the short-lived radical gas nitric oxide, or other reactive nitrogen intermediates (hereafter refered to generically as NO[1]), generated via enzymatic oxidation of a guanidino nitrogen of L-arginine in a wide variety of mammalian cells, is a potentially important regulator of the cardiovascular, nervous, and immune systems (1). In activated mouse macrophages such products have antitumor activity, and production of NO has been implicated as an antimicrobial effector mechanism both extracellularly (*Cryptococcus neoformans* [2]; schistosomula of *Schistosoma mansoni* [3]) and within the cell (amastigotes of *Leishmania major* [4]; trophozoites of *Toxoplasma gondii* [5]; *Trypanosoma musculi* [6]; *Mycobacterium leprae* [7]; *Mycobacterium tuberculosis* [8]). However, in the human macrophage, a cell with well-developed antitumor and antimicrobial capacities, the question of immunologically inducible NO production is controversial (9–11).

Human polymorphonuclear leukocytes (neutrophils; PMN) secrete NO (12), and roles for NO have been proposed in PMN chemotaxis (13), in the regulation of PMN adhesion to endothelium (14), and in the elaboration of PMN-dependent tissue damage (15). NO did not appear implicated in killing of staphylococci by PMN (13), perhaps, we thought, because intact PMN are so heavily armed with other killing mechanisms (16) that this one is not normally observed. In studying motile, anucleate fragments from human PMN (cytokineplasts; CKP) (17–19), we were struck by their considerable residual bacterial killing capacity despite having few or no granules and no activatable respiratory burst oxidase activity (20–22). We now describe classical evidence for rapid killing of staphylococci by CKP via NO: inhibition of killing by the competitive inhibitor of nitric oxide synthase, N^{ω}-monomethyl-L-arginine (NMMA); restoration of killing by the addition to NMMA of excess L-arginine; and no restoration with D-arginine. We propose that the generation of NO serves as an additional microbial killing pathway in PMN, and that cytoplasts can be used to help delineate the spectrum of susceptible targets.

Methods

Reagents. NMMA, L-arginine, and D-arginine were from Sigma Chemical Co., St. Louis, MO.

Preparation of cells. Human blood leukocytes were prepared as described previously (23) from heparinized venous blood. CKP were produced by brief heating (45°C, 9 min) of human peripheral blood PMN on surfaces, and separation from their cell bodies by discontinuous density centrifugation (17, 24). U-CYT were prepared by discontinuous gradient centrifugation without cytochalasin B (22). Both fragments were cryopreserved until use (25).

Phagocytosis assay. The phagocytosis system was as described previously (23) except for smaller volumes. Briefly, cytoplasts or PMNs were preincubated for 15 min with or without agents in Krebs-Ringer phosphate buffer with glucose (KRPG) and 11% pooled human serum at

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1. *Abbreviations used in this paper:* CKP, cytokineplasts; KRPG, Krebs-Ringer phosphate buffer with glucose; NMMA, N^{ω}-monomethyl-L-arginine; NO, nitric oxide or other reactive nitrogen intermediates; Staph, *Staphylococcus aureus* strain 502A; U-CYT, U-cytoplasts.

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37°C in a shaking water bath before the addition of *Staphylococcus aureus* strain 502A (Staph) (that had been grown up overnight) at a ratio of 15–38:1, Staph:CKP or PMN. Duplicate samples were removed at 20 and 60 min, centrifuged, the supernatants reserved, the pellets disrupted, and live bacteria from supernatants and pellets were quantified by duplicate plating in agar. 60-min samples were also allowed to settle onto cover slips pretreated with Alcian blue to promote cell adhesion (26) (30 min, 37°C), air dried, and stained with Wright-Giemsa for direct viewing by phase-contrast microscopy (25).

Acridine orange determination of bacterial viability. 20-min samples were allowed to settle onto Alcian blue–coated coverslips (20 min, 37°C), and nonadherent cells were gently washed away with prewarmed KRPG/10% autologous serum. The living, adherent cytoplasts were stained with acridine orange at 2×10^{-6} M for 45 s, washed in KRPG, mounted onto glass slides, sealed with paraffin, and imaged live using a confocal imaging system (MRC 600; Bio-Rad Laboratories, Richmond, CA) linked to a microscope (Axiovert 10; Carl Zeiss Inc., Thornwood, NY).

Electron microscopy. Samples were prepared as for the phagocytosis assay (above) and processed as described previously (27). After 60 min in suspension, cytoplasts were fixed in 2% glutaraldehyde, pelletted, postfixed in 2% OsO_4, stained with 3% uranyl acetate, dehydrated in ethanol, embedded in Epon 812 (Ernest F. Fullam, Inc., Latham, NY), and examined on an electron microscope (301; Phillips Electronic Instruments Co., Mahwah, NJ).

Results

Effect of NMMA on uptake and killing of staphylococci by cytokineplasts (CKP: Figs. 1 and 2). The NO synthase inhibitor NMMA produced a modest decrease in uptake of bacteria by CKP from supernatants, and a marked decrease in the killing of cytoplast-associated bacteria. After 20 and 60 min of incubation, control supernatants from seven experiments (Fig. 1 *A*) contained 17%±2.0% (mean±SEM) and 15%±1.3%, respectively, of the inocula, versus 32%±3.7% and 30%±4.2%, respectively, for NMMA-treated CKP (for both intervals, $P < 0.02$).

Live, CKP-associated bacteria at the two intervals (Fig. 1 *B*) were 16%±1.5% and 14%±1.3%, respectively, for controls, and 50%±3.0% and 51%±3.6%, respectively, for NMMA-treated CKP (for both intervals, $P < 0.001$). Both effects were essentially complete by the first sampling interval (20 min), and even after 60 min of incubation, NMMA-treated cytoplasts had a mean of over 3.5 times as many live, CKP-associated bacteria as did controls, despite having taken up fewer.

The mean total percentages of bacteria killed by 60 min (Fig. 1 *C*), (100 − [mean percent live supernatant bacteria + mean percent live cytoplast-associated bacteria]), are 71%±2.1% for controls versus 19±4.7% for NMMA-treated CKP ($P < 0.001$). The 20-min values (not shown) are similar, and equally significant statistically.

Although L-arginine had no stimulatory effect on uptake or killing of bacteria by CKP, it reversed the inhibitory effect of NMMA (Fig. 1). This reversal is also seen qualitatively in live, adherent CKP stained with the fluorescent dye, acridine orange, which distinguishes live from dead bacteria (28) (Fig. 2). The images shown here are 0.5-μm thick *z*-axis optical sections obtained using scanning laser confocal microscopy. In addition to optical sectioning, confocal microscopy allows visualization of two fluorescent wavelengths simultaneously, allowing in this case the demonstration of both live (green) and dead (red) CKP-associated bacteria. With time, the living, NMMA-treated cytoplasts (Fig. 2 *A*) have become flatter and therefore appear larger; staining of large numbers of bacteria, seen in a single plane, is predominantly green. Addition of L-arginine to NMMA allows killing to proceed (Fig. 2 *B*); these CKP are less flat, and the red-staining CKP-associated material appears larger than individual bacteria, suggesting superimposition or fusion of phagosomes. These latter CKP resemble untreated CKP given bacteria (not shown).

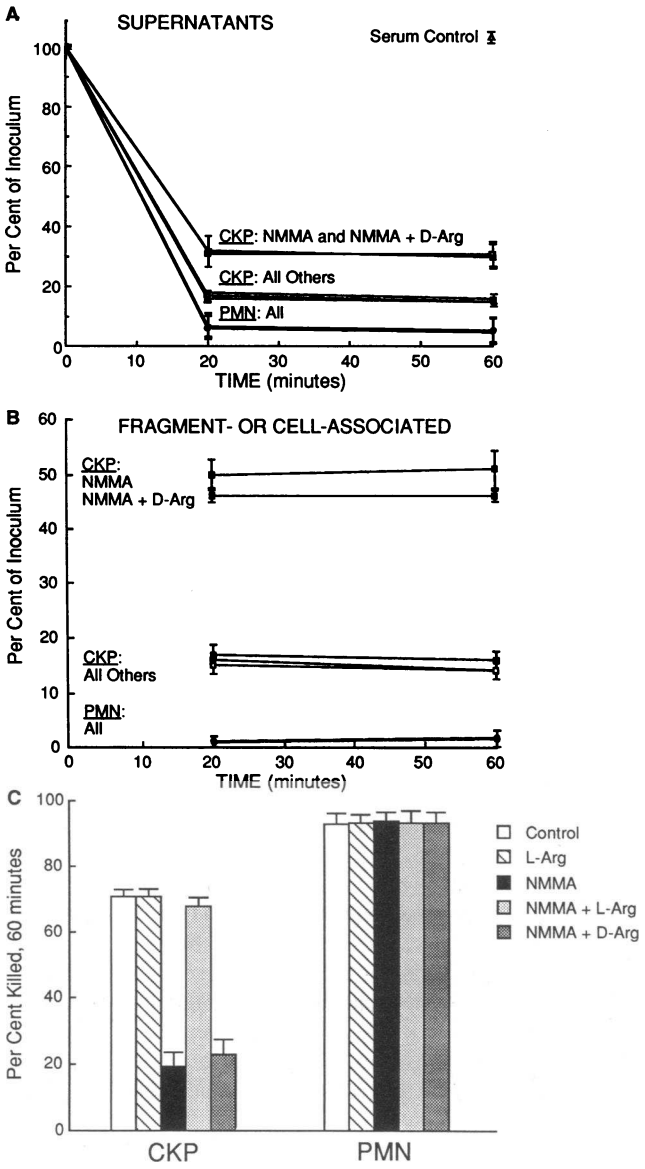

Figure 1. Effect of NMMA on uptake and killing of staphylococci by CKP and by intact PMN. Results have been combined from seven separate experiments in which, after preincubation of CKP for 15 min with or without agents, Staph were added and duplicate samples taken after 20 min of further incubation, and again after 60 min; means±SEM are indicated. (*A*) NMMA-treated CKP remove bacteria from the medium less efficiently than control CKP ($P < 0.02$, both intervals), and (*B*) are much less efficient at killing what they have removed ($P < 0.001$). Both effects are reversed by L-arginine but not by D-arginine. (*C*) Percent Staph killed at 60 min is derived from the line drawings: 100 − (supernatant + CKP-associated). Intact PMN (two experiments) are more efficient at both uptake and killing than control CKP, and are unaffected by NMMA. Mean ratio, Staph:CKP was 29:1 (range, 15–38:1); ratios Staph:PMN were 31:1 and 33:1. Agents added were 1 mM NMMA, 10 mM L-arginine, 1 mM NMMA + 10 mM L-arginine, 1 mM NMMA + 10 mM D-arginine.

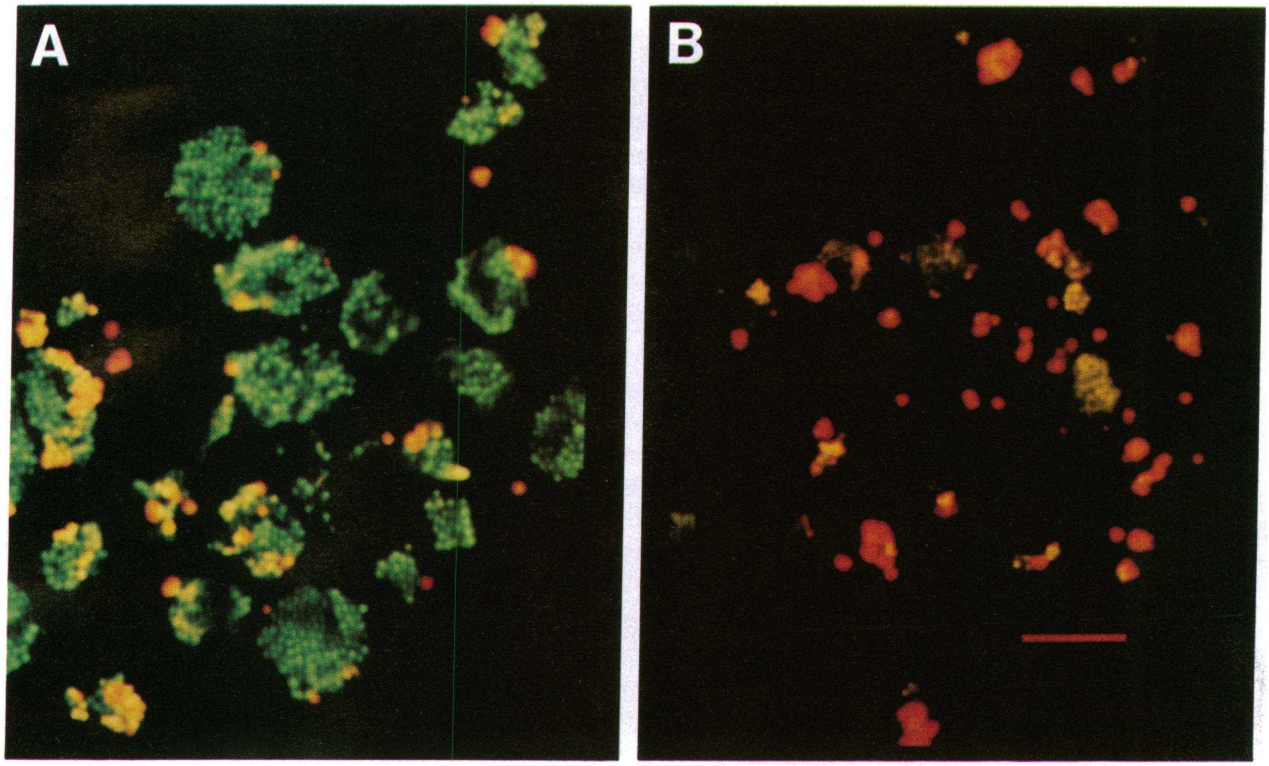

Figure 2. Effect of NMMA on killing of staphylococci by CKP. CKP were preincubated as described in Fig. 1. After 20 min of incubation with Staph, CKP were plated onto glass slides, incubated 20 min at 37°C, stained with acridine orange, and imaged live using a confocal imaging system (see Methods). Live Staph appear green; dead ones, red. Cytoplasts were pretreated with (*A*) 1 mM NMMA, and (*B*) 1 mM NMMA + 10 mM L-arginine. Those in (*B*) resemble untreated controls. Bar = 10 μm.

In fixed and stained specimens viewed in phase-contrast microscopy, where CKP borders are clearly visible, bacteria do not appear adherent to the periphery of cytoplasts, whether the latter are untreated or have been treated with NMMA, or NMMA and L-arginine (Fig. 3). As with intact PMN, numbers of bacteria per cytoplast vary (25). Similarly, CKP prepared in suspension, fixed, cut in thin sections, and viewed in the electron microscope, again resemble each other regardless of treatment. Specifically, one sees bacteria primarily in vacuoles, not adherent to plasmalemma (Fig. 4).

Lack of effect of D-arginine on NMMA-treated CKP. In contrast to L-arginine, D-arginine did not reverse the inhibition of either uptake or killing (Fig. 1). In four of seven experiments in which D-arginine was used, the mean killing by NMMA-treated CKP at 20 and 60 min was 21%±5.9% and 23%±7.4%, respectively, versus 24%±5.2% and 23%±4.7% for CKP treated with both NMMA and D-arginine. D-arginine alone had no effect (not shown).

Effect of NMMA on uptake and killing of staphylococci by PMN (Fig. 1). Intact PMN take up and kill bacteria more efficiently than do CKP (20, 25). In the two experiments shown in Fig. 1, NMMA had no effect on uptake or killing of bacteria. Control values at 20 and 60 min versus values for NMMA-treated preparations were as follows: supernatants contained 6%±3.9% and 5%±4.8%, respectively, of the inocula, versus 7%±4.5% and 5%±4.4%; cell-associated values were 1%±0.4% and 2%±1.2%, respectively, versus 1%±0.4% and 2%±1%; and percent killing at the two intervals was 93%±3.5% and 93%±3.6%, respectively, versus 92%±4.1% and 94%±3.4%.

We saw no effect of NMMA on PMN even at 10 times the molar concentration used for CKP (not shown). To address the unlikely possibility that intact PMN could deplete the inhibitor, we applied supernatants from NMMA-treated PMN to CKP; their inhibitory properties, reversible with L-arginine, were still present.

Effect of NMMA on uptake and killing of staphylococci by U-CYT and by PMNCGD. (Fig. 5). Because CKP lack activatable oxidase, and because superoxide (O_2^-) and NO can scavenge each other (29, 30), we wondered whether the failure of NMMA to affect PMN might be due to a killing hierarchy in which reactive oxygen products predominate over NO. To test that hypothesis, we used U-CYT (granule poor, but unlike CKP, retain activatable oxidase [22, 25]), as well as PMN from a well-characterized patient with CGD (31), which are totally without activatable oxidase activity. In two experiments, uptake and killing of bacteria by U-CYT were inhibited in a manner indistinguishable from that seen in CKP (Fig. 5). In contrast, the already limited killing of bacteria by the PMNCGD was unaffected by NMMA; i.e., they behaved in this respect like normal PMN (Fig. 5). Thus, NMMA inhibited uptake and killing of bacteria in both of the granule-poor cytoplasts and in neither of the granule-rich cells, without regard to the presence or absence of activatable oxidase.

Discussion

The NO synthase inhibitor and L-arginine analogue NMMA produced a decrease in uptake and killing of staphylococci by

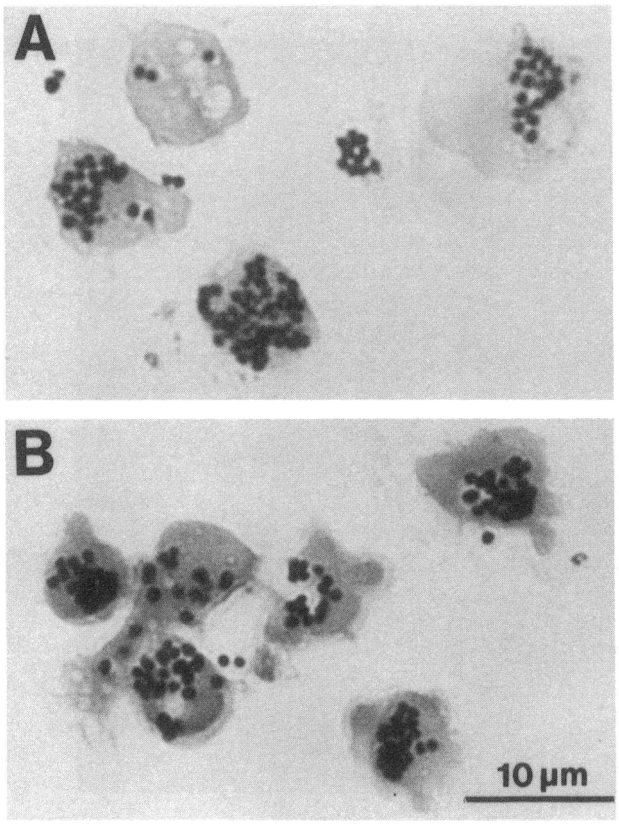

Figure 3. Cell-associated bacteria: phase contrast. The experimental design is as in Fig. 1. After 60 min of incubation with bacteria in suspension, samples were allowed to adhere to glass and were dried, stained, and viewed directly by phase-contrast microscopy (see Methods). Cytoplasts were pretreated with (*A*) 1 mM NMMA, (*B*) 1 mM NMMA + 10 mM L-arginine, or neither (not shown). (*A*) and (*B*) resemble both each other and untreated controls; variable numbers of bacteria are associated with cytoplasts in which vacuolization is often visible.

CKP derived from human PMN. These effects, essentially complete at the first sampling interval (20 min), were reversible by L- but not by D-arginine (Figs. 1 and 2). Even after 60 min of incubation, NMMA-treated cytoplasts had a mean of over 3.5 times as many live, CKP-associated bacteria as did controls (51% of the inocula versus 14%). These findings provide the first evidence linking microbial killing to NO production in PMN from any animal.

Note that the quantitative data are expressed as uptake of bacteria by cytoplasts, and killing of cytoplast-associated bacteria. This terminology does not distinguish adherent from ingested bacteria, but we strongly suspect that they are largely intracellular because (*a*) bacteria adherent to plasmalemma were not notable either by phase-contrast (Fig. 3) or electron microscopy (Fig. 4), and (*b*) if decreased killing in the presence of NMMA were significantly due to a slowing down of the internalization of bacteria, we would not expect essentially the same recoveries of supernatant and of cytoplast-associated bacteria at 20 and again at 60 min.

Since NO synthase appears to be functional in activated PMN (12–14), and there is no evidence for scavenging of NO or of depletion of NMMA by PMN during phagocytosis (above), the most likely explanation for why effects of NMMA on uptake and killing of staphylococci are not observed in intact cells is perhaps our original hypothesis: granule-rich intact PMN are so well endowed with killing substances (16) that the NO killing pathway is not ordinarily in evidence. In this regard it is worth noting that the PMNCGD employed here killed staphylococci better than those from most patients with chronic granulomatous disease; they were chosen for their defined defect and total absence of activatable oxidase activity (31). In cells with more severe functional impairment, the NO pathway may be both visible and important for survival; this deserves further study.

With NO assigned a likely role in bacterial killing, CKP and U-CYT, as "stripped-down" PMN, should be excellent re-

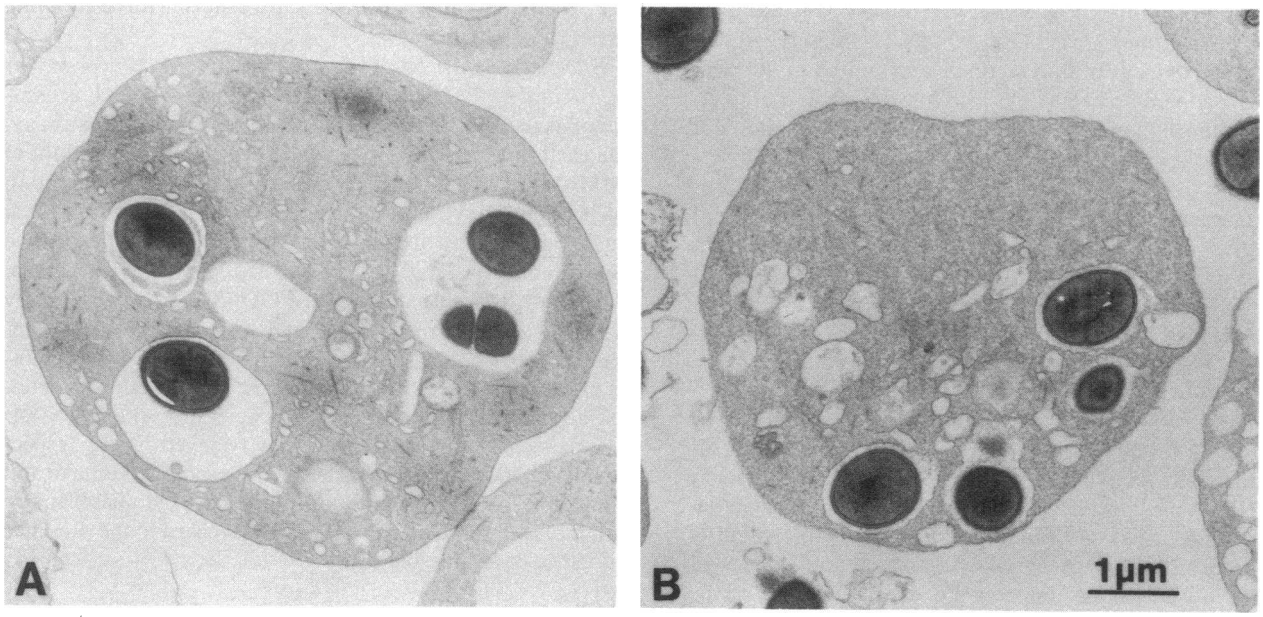

Figure 4. Cell-associated bacteria: thin sections. The experimental design is as in Fig. 1. After 60 min of incubation with bacteria in suspension, samples were prepared for electron microscopy (see Methods). Cytoplasts were pretreated with (*A*) 1 mM NMMA, (*B*) 1 mM NMMA + 10 mM L-arginine, or neither (not shown). As in Fig. 3, (*A*) and (*B*) resemble both each other and untreated controls; bacteria appear primarily within vacuoles, rather than adherent to plasmalemma.

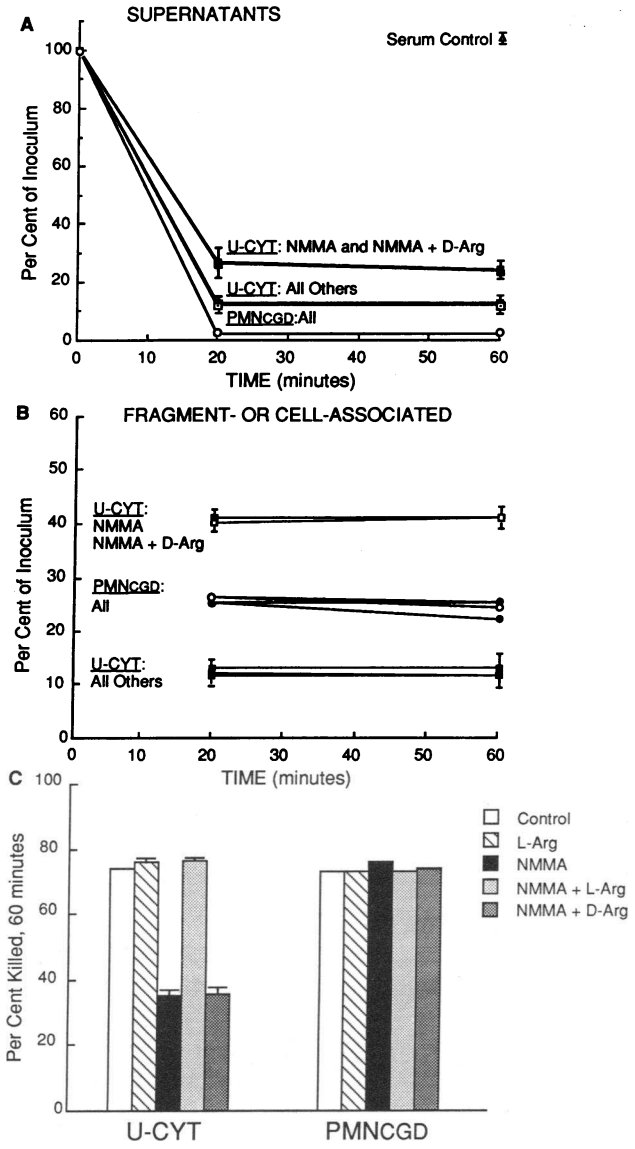

Figure 5. Effect of NMMA on uptake and killing of staphylococci by U-CYT and by intact PMNCGD. The experimental design is as in Fig. 1 except that we used U-CYT (two experiments), which, unlike CKP, retain activatable oxidase, and intact PMNCGD, which lack activatable oxidase. Again L-arginine–reversible inhibition of uptake and killing of Staph was seen with the granule-poor cytoplasts but not with the granule-rich intact PMN; inhibitory effects of NMMA seem therefore unrelated to the presence or absence of activatable oxidase. The ratios of Staph:U-CYT were 16:1 and 27:1; ratio Staph:PMNCGD was 23:1.

porter cells for learning which microorganisms are susceptible to killing by NO. We already know, for example, that *Candida* hyphae are resistant to killing by U-CYT unless granule products are added back (Stein, D. K., S. E. Malawista, G. Van Blaricom, and R. D. Diamond, submitted for publication); NO is therefore not likely to have a significant role in their disposal. Granule-poor cytoplasts should teach us a great deal about the range of this new killing pathway.

Acknowledgments

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