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MUTUAL CONTACT OF ADHERENT POLYMORPHONUCLEAR LEUKOCYTES INHIBITS THEIR GENERATION OF SUPEROXIDE

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Superoxide generation by polymorphonuclear leukocytes (PMNs) in suspension, or adherent to glass or plastic, after stimulation with *N*-formylmethionyl-leucyl-phenylalanine or phorbol myristate acetate was measured by cytochrome c reduction and spin trapping. Amounts of superoxide generated by adherent PMNs were inversely related to cell density. The generation of hydrogen peroxide was also inhibited at higher cell densities. In contrast to adherent cells, superoxide released by PMNs in suspension linearly increased with respect to cell number over a wider range. Microscopic observation indicated that the number of cells in mutual contact increased rapidly at cell densities higher than 4×10^4 cells/cm^2, and inhibition of superoxide became apparent at higher cell densities. Mediators which could be released by PMNs, such as NO and adenosine, were not the cause of inhibition. These data suggest that mutual contact of PMNs suppresses their generation of superoxide. Survival rates of PMNs after stimulation increased at higher densities, indicating that the mutual contact-induced inhibition of superoxide generation by PMNs may be physiologically relevant at sites of inflammation.

KEY WORDS: Cell adhesion, Cell density, Hydrogen peroxide, Cell survival, Electron paramagnetic resonance, Spin trapping.

INTRODUCTION

Polymorphonuclear leukocytes (PMNs) accumulate at sites of bacterial infection through adhesion to endothelial cells, diapedesis and chemotaxis. At the foci of infection, PMNs destroy bacteria by means of various factors which include active oxygen species. These factors are toxic not only to microorganisms but also to nearby host cells and to PMNs themselves.[1,2] In order to avoid host cell damage, there must be mechanisms which specifically select targets of attack. One example that suggests this possibility is that the respiratory burst of PMNs is inhibited on closed monolayer cultures of endothelial cells.[3] Like endothelial cells, PMNs themselves might have a mechanism which prevents them from attacking each other.

There have been discrepant reports when rates of superoxide generation by adherent PMNs were compared to those from PMNs in suspension.[4-9] These discrepancies might be explained by differences in cell density in various studies.

Experiments described in this report indicate that the amount of superoxide from

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PMNs was proportional to cell populations over a wide range when PMNs were in suspension, whereas when PMNs were stimulated in adhesion, the amounts of superoxide released per unit cell decreased with increases in the cell number. Microscopic observation indicated that rates of superoxide generation were inversely related to the number of cells in mutual contact. These observations may indicate that mutual contact of PMNs down-regulates superoxide generation.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase (HRP) was purchased from Boehringer Manheim (Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO). The final concentrations of the following were used in reaction mixtures: *N*-formylmethionylleucylphenylalanine (fMLP, 10^{-5} M), phorbol myristate acetate (PMA, 100 ng/ml), diethylenetriaminepentaacetic acid (DETAPAC, 10^{-4} M), 5,5-dimethylpyrroline-*N*-oxide (DMPO, 0.1 M), cytochrome c (10^{-4} M).

Leukocyte Isolation and Fixation

Heparinized venous blood was obtained from healthy human volunteers, and PMNs and mononuclear cells were isolated after methods of Ferrante et al.[10,11] A mixed solution of meglumine diatrizoate and sodium diatrizoate at a ratio of 2:1 was used as a substitute for Hypaque®. After hypotonic lysis of contaminating erythrocytes, PMNs were washed with Hanks' balanced salt solution (HBSS) and were suspended in the same medium. Cells from the mononuclear cell layer were incubated in a petri dish at 37°C for 30 min, and lymphocytes were isolated as non-adherent cells. The following experiments were conducted at room temperature (20 ± 2°C). Some PMNs were incubated in a 1% paraformaldehyde solution of HBSS for 4 hr. After washing with HBSS twice, the fixed PMNs were suspended in HBSS.

Proportion of Adhesion

PMNs were allowed to settle on the plastic surface of 24-well or 6 -well culture petri dishes (Falcon) during a 30 min incubation. The hydrophilic property of the wall of the well made fluid surfaces concave, resulting in an extremely uneven cell distribution at the bottom. In order to overcome the problems with the concave surface, plastic sheaths which have more hydrophobic surfaces were placed in the wells. The sheaths were prepared by cutting polypropylene culture tubes (Fisher Scientifics, outer diameter: 13 mm), and were used after sterilization by autoclaving. PMN suspensions were gently introduced into the sheaths. After 30 min incubation the sheaths and supernatants were carefully removed. PMNs were evenly distributed on the bottom of wells only inside the area of the sheaths.

The proportion of adherent PMNs which were resistant against gentle exchange of the media was determined by measuring the protein remained in petri-dishes. After removing the supernatant carefully, protein on the plates was measured as described by Lowry et al., using bovine serum albumin as a standard.[12] The proportions of adhesion were calculated by dividing the protein amounts with those of added PMNs.

Cytochrome c Reduction Assay and H_2O_2 Assay

After PMNs were allowed to settle as described above, the medium was replaced with 600μl HBSS containing cytochrome c, DETAPAC, and fMLP or PMA. After incubation for 15 min, 500 μl supernatant was diluted with an equal volume of HBSS, and the absorbance at 550 nm was measured (Perkin Elmer Lambda 3 Spectrophotometer). Superoxide dismutase (SOD, from bovine erythrocytes, 100 μg/ml) was added to some wells to test for superoxide-independent cytochrome c reduction. When 6-well plates were used, the volume of the reaction mixture was 1 ml instead of 600 μl, and the absorbance of the supernatant was measured without dilution. Superoxide from PMNs in suspension was measured using the same reaction mixture. H_2O_2 released from adherent PMNs was assayed utilizing the shift of the absorbance peak of HRP after formation of compound II.[13] In this assay, sheaths were prepared as described above except that 50 ml centrifuge tubes (Fisher Scientifics) were used. PMNs settled at various densities on 6-well culture plates inside the sheaths during 30 min of incubation. The sheaths and supernatant were carefully removed and replaced by 1 ml of HBSS containing HRP (10 μM), sodium azide (1 mM), DETAPAC and fMLP. Sodium azide was used to inhibit catalase and myeloperoxidase activities of PMNs. The absorbances at 417 nm and at 413 nm of the supernatant were measured after 3 min incubation. Concentrations of H_2O_2 were calculated using $\varepsilon = 50{,}000$ for $\Delta(OD_{417}-OD_{413})$.[14]

Spin Trapping Experiments

The spin trapping agent used in these experiments was DMPO, which forms radical adducts (spin adducts) with both superoxide and hydroxyl radicals. DMPO was purified by the method reported previously.[9] A PMN suspension was introduced into an EPR flat cuvette (Wilmad WG814), placed horizontally and incubated for 10 min to allow the cells to settle. After gentle washing with 500 μl of HBSS, a solution of DMPO, DETAPAC and fMLP or PMA dissolved in HBSS was introduced to the cuvette. EPR spectra were recorded using a Bruker ER300E EPR spectrometer equipped with 100 kHz field modulation. The cuvette was placed in the EPR cavity in a horizontal direction. Other typical EPR operating conditions were: microwave power, 20 mW; modulation width, 0.1 mT; field sweep, 10 mT/84 s; time constant, 160 ms.

The percent of adherent PMNs in contact with other PMN(s) at various cell densities was measured using EPR flat cells. After PMNs were allowed to adhere to the wall of a cuvette as described above, the number of total cells as well as the number of cells in contact with other cell(s) in a unit area of the cuvette surface were counted using an inverted microscope (Olympus) equipped with an eyepiece micrometer.

Survival Rates of PMNs

Various number of PMNs were allowed to settle on 24-well plates using sheaths as described above. After 30 min of incubation, the sheaths and the supernatants were carefully removed, and replaced with HBSS containing DETAPAC and fMLP. The number of cells which excluded trypan blue in a unit square were counted after various intervals using an inverted microscope.

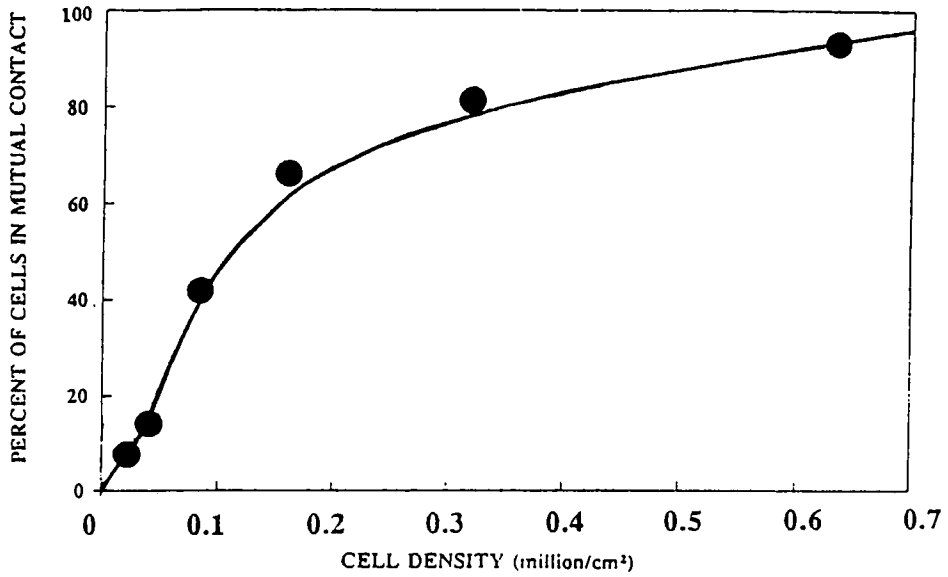

FIGURE 1 The percent of adherent PMNs in contact with other PMN(s) at various cell densities. Various concentrations of PMNs in suspension were introduced into flat EPR cells, and the number of total cells, as well as cells having mutual contact in a unit area were counted using a inverted microscope. Each point represents the mean of at least 3 measurements at different areas.

RESULTS

Mutual Contact of PMNs

The percent of PMNs in mutual contact was counted after the cells were allowed to adhere to an EPR cuvette. When the density of PMNs was lower than 4×10^4 cells/cm^2, less than 15% of the adherent cells were in mutual contact with each other. As the density increased, the ratio of cells which were in contact with other cell(s) increased, and at the density of 6.4×10^5 /cm^2, 93% of total PMNs had contact with other PMN(s) (Figure 1).

Proportion of Adhesion

Microscopic observation revealed that most PMNs were adherent on the bottom of petri-dishes after 30 min incubation, and were resistant against gentle removal of supernatant medium. As measured by protein content, less than 2% of PMNs were lost after gentle exchange of media at any PMN concentration, up to twice as high as that used in the superoxide assay (data not shown).

Superoxide Generation by the Same Number of PMNs at Different Densities

Four sheaths were placed in each well of 6-well plates, and the same total number of PMNs (6.4×10^5) were introduced into each well. In Group 1, all PMNs were placed in one of the four sheaths (Figure 2). In Group 2 and Group 3, PMNs were divided equally into two and four sheaths, respectively (Figure 2). The resulting densities of

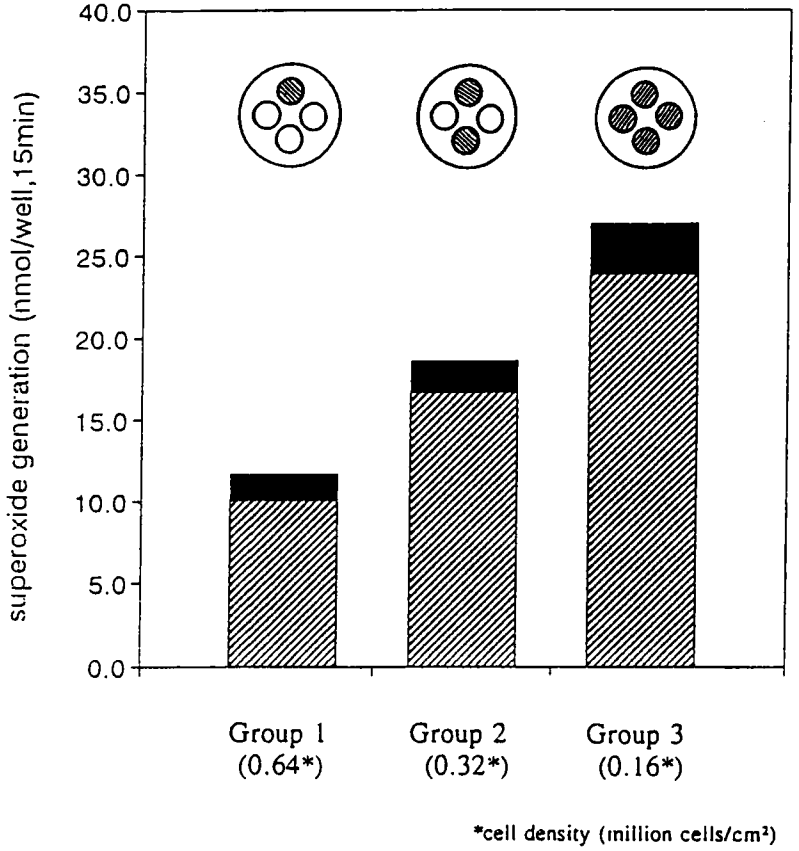

FIGURE 2 Superoxide generation by the same number of PMNs at different densities. Four sheaths (inner area: 1.0cm^2) were placed in each well. PMN suspensions (6.4×10^5/well) were introduced into one (Group 1), or two (Group 2), or all (Group 3) of the four sheaths. The large circles in the figure illustrate wells, and the small circles in the large ones illustrate the sheaths in the wells. Sheaths in which PMNs were introduced are indicated by oblique stripes. After the cells settled, SOD inhibitable cytochrome c reduction during 15 min after fMLP (10^{-5} M) addition was measured. The bar height and closed area indicate the mean and s.d. of three experiments. The differences between each group were statistically significant by Student t test ($p<0.01$).

cells were 6.4×10^5 /cm^2, 3.2×10^5 /cm^2 and 1.6×10^5 /cm^2, respectively. The amounts of superoxide measured by SOD inhibitable cytochrome c reduction are shown in Figure 2. Although the total number of PMNs in each well was the same, superoxide generation was suppressed at higher densities.

Using the spin trapping technique, similar results were obtained. The cell density was altered by changing the number of surfaces of a flat EPR cell on which PMNs were distributed. Bilateral distribution (PMNs were distributed on the two inside surfaces of a cuvette) was achieved by introducing PMN suspensions twice with a 10 min interval and rotating the flat cell 180 degree before the second introduction. PMNs introduced into a horizontally placed EPR flat cuvette adhered approximately 95% to the bottom flat surface after 5 min incubation. Unilateral distribution was attained by the same manner as above omitting the reversal of the cuvette. The resulting density of PMNs were 1.6×10^5 /cm^2 and 3.2×10^5 /cm^2, respectively. In either experiments, after an

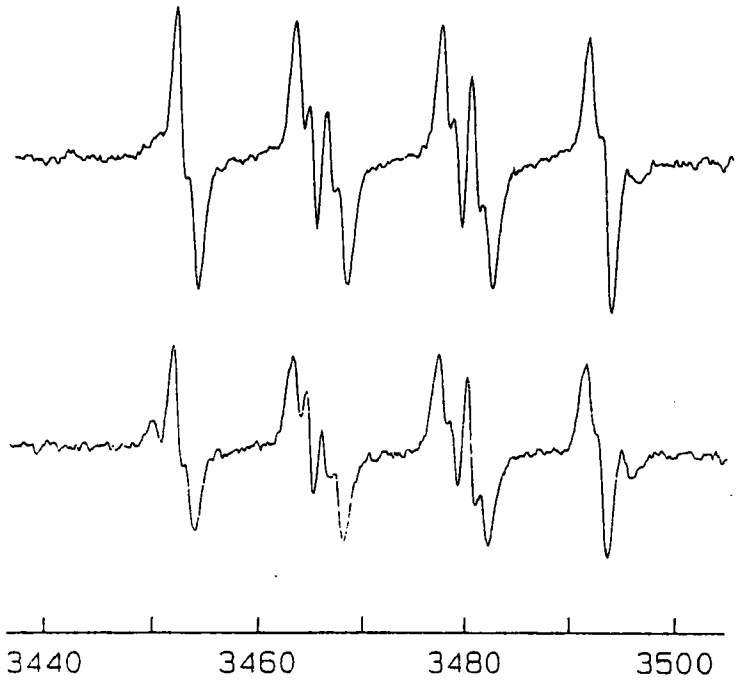

FIGURE 3 The EPR spectra of DMPO spin adducts obtained by fMLP-stimulated PMNs. The top: PMNs were distributed on both sides of an EPR cuvette at a density of 0.32×10^6/ cm^2. The bottom: PMNs were distributed on one side of the cuvette at a density of $0.64\times10^6/cm^2$. In either experiment, the total number of cells in the cuvette was the same.

additional 10 min (total 20 min) of incubation, HBSS containing DMPO, DETAPAC and fMLP was introduced, and EPR spectra were recorded intermittently. Counts of adherent cells did not decrease by washing and replacement with fresh buffer.

One minute after a buffer solution of DMPO (100 mM) and fMLP was introduced to the cuvette, EPR spectra shown in Figure 3 were obtained. The spectra were assigned to two radical species *i.e.*, superoxide adduct of DMPO (DMPO-OOH) and hydroxyl adduct of DMPO (DMPO-OH), but the origin of the DMPO-OH adduct is controversial.[15,16] Because the aim of this study is to evaluate superoxide generation by PMNs, only DMPO-OOH signals will be discussed further. The DMPO-OOH signal of the sample, when PMNs were distributed on only one side of the cuvette, was smaller than when PMNs distributed on both sides (Figure 3). The signal intensity one minute after stimulation when the PMN density was 3.2×10^5 /cm, was 73% of that when PMN density was 1.6×10^5 /cm, and this ratio was comparable to that obtained by the cytochrome c assay: 69% (Figure 2).

Superoxide and H_2O_2 Generation by Different Numbers of PMNs in the Same Area

Cytochrome c reduction was measured from various numbers of PMNs using 24-well plates. As indicated in Figures 4 and 5, the dose-response relation showed a saturation either with fMLP-stimulation or with PMA-stimulation. These results indicate that the efficiency of superoxide generation decreased as the density of adherent PMNs increased. In contrast, when the experiments were conducted with PMNs in suspension

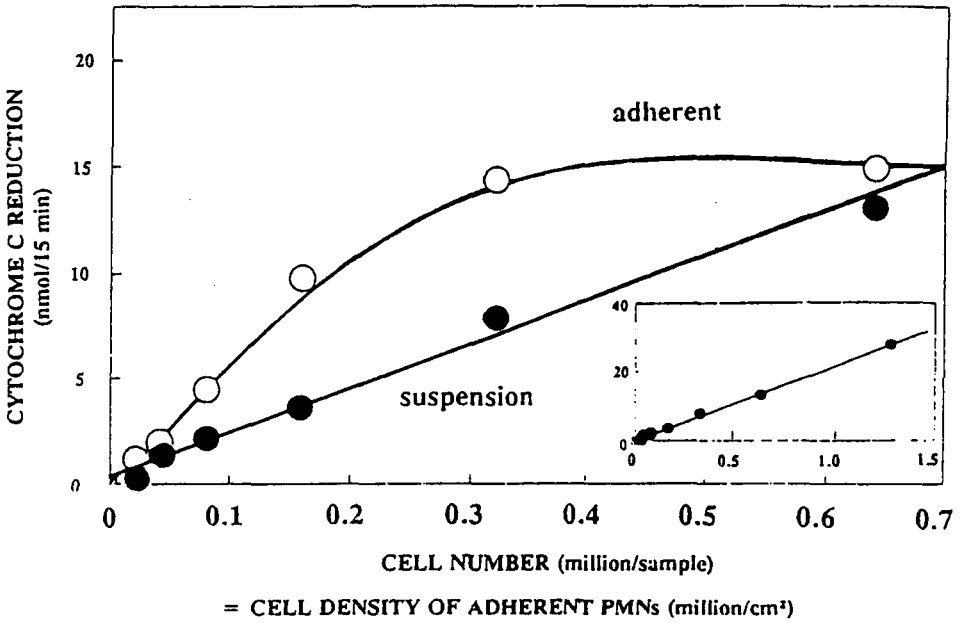

FIGURE 4 Cytochrome c reduction by fMLP-stimulated PMNs as a function of cell number. ●, PMNs in suspension; ○, PMNs in adhesion. In adherent cells, cell number is equal to area cell density, because the distribution area was 1 cm^2. The inset shows a wider range view of the results using PMNs in suspension. Values are means from at least three measurements with PMNs from the same donor. Preparations from different donors always showed a similar pattern.

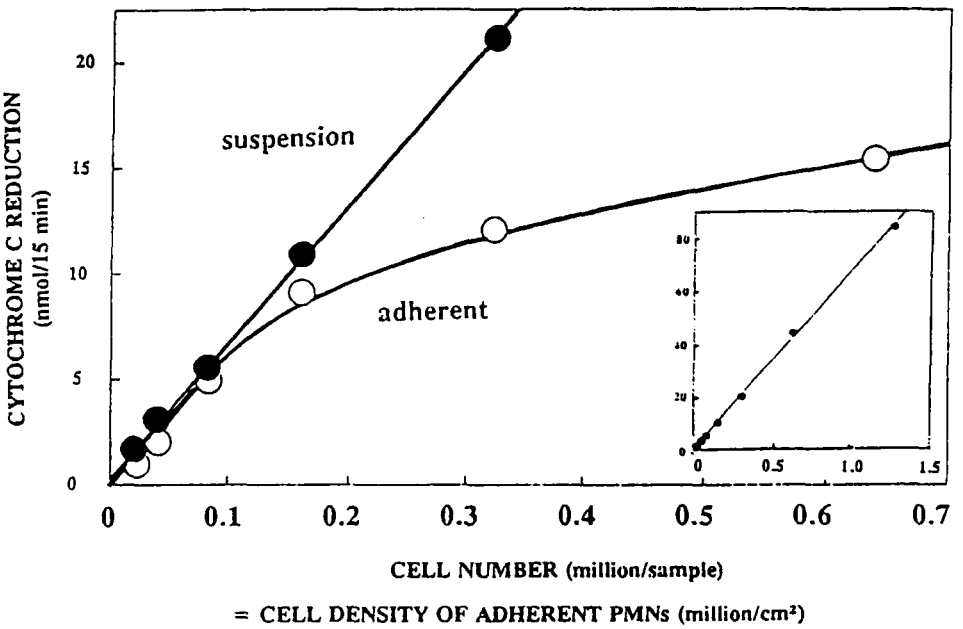

FIGURE 5 Cytochrome c reduction by PMA-stimulated PMNs as a function of cell number. Other conditions as in Fig. 4.

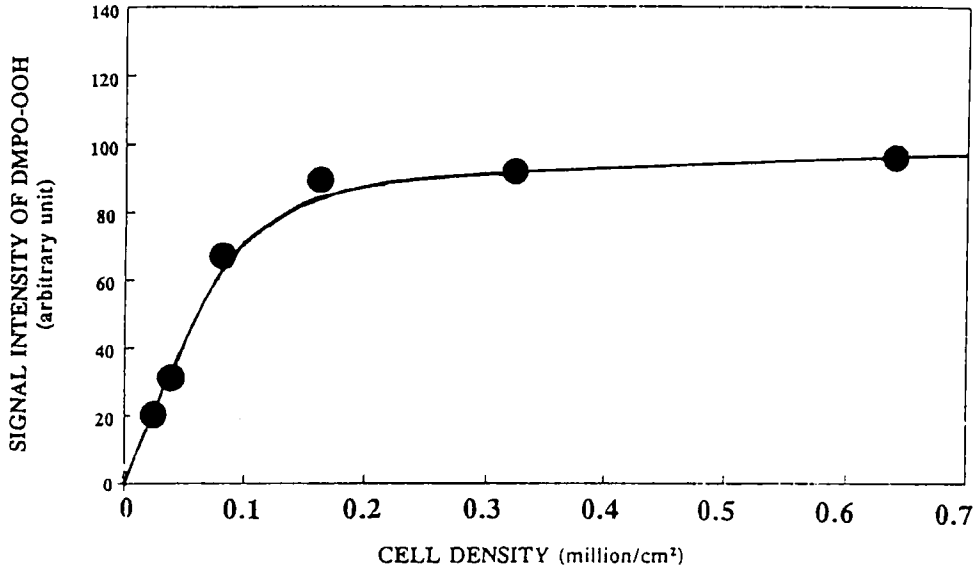

FIGURE 6 The DMPO-OOH signal intensity as a function of PMN density. Each point represents the mean of at least 2 experiments using the same preparation of PMNs. Several sets of experiments using different preparations of PMNs always showed the same pattern.

in an identical volume of reaction fluid (600 μl), superoxide generation was proportional to the amounts of cells in a wider range. At the density of 0.64×10^6 cells /cm^2, adherent PMNs occupied almost all surface area, thus higher densities were not tested for adherent PMNs. The results of spin trapping experiments are shown in Figure 6. The relationship between the signal intensity of DMPO-OOH 1 min after stimulation and the cell density was almost the same as that obtained by the cytochrome c assay.

H_2O_2 generated from various numbers of PMNs is shown in Figure 7. The dose-response curve was similar to that of superoxide generation (Figures 4, 5, 6).

Survival of PMNs at Various Cell Densities

PMNs adherent on a petri dish and stimulated with fMLP gradually disappeared by lytic cell death. Most of the remaining PMNs excluded trypan blue. The survival curve obtained by trypan blue exclusion is shown in Figure 8. Higher survival rates were observed with PMNs at higher densities.

Effect of Lymphocytes and Fixed PMNs

In order to determine whether inhibition requires contact between viable cells, PMNs were incubated with fixed PMNs. PMNs were allowed to settle at a density of 3.2×10^5 /cm^2 on 24-well plates using the sheaths as described above. PMNs from the same donor which had been fixed by paraformaldehyde were added to the reaction mixture of the cytochrome c reduction assay at the ratio of two fixed PMNs per live PMNs. As shown in Table 1, fixed PMNs had no inhibitory effect on superoxide generation. In order to determine whether the inhibition by cellular contact is cell linage specific, PMNs were incubated with lymphocytes from the same blood sample. Lymphocytes were added to

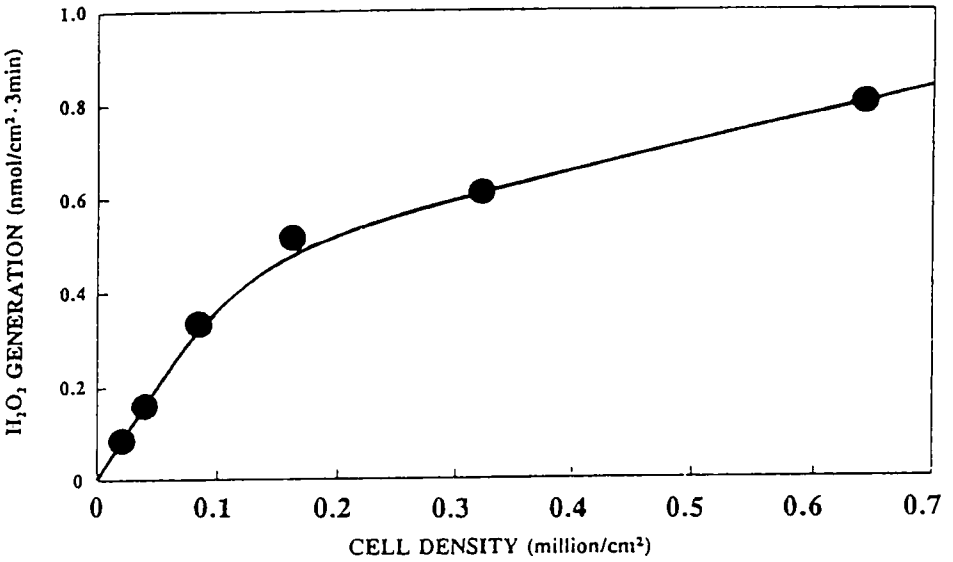

FIGURE 7 H_2O_2 generation by adherent PMNs after stimulation with fMLP as a function of PMN density. H_2O_2 was measured using the shift of HRP absorbance when compound II was formed. Each point represents the mean of at least 3 experiments using the same preparation of PMNs. Several sets of experiment using different preparations of PMNs always showed the same pattern.

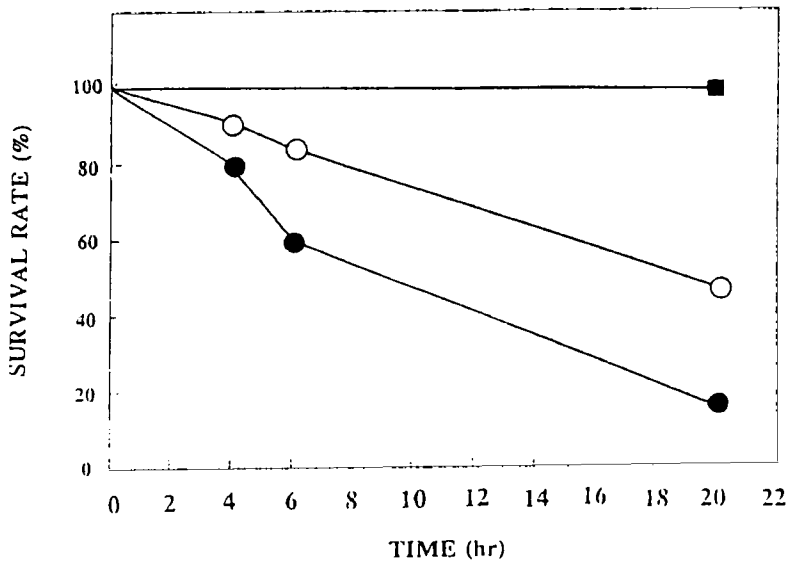

FIGURE 8 The survival of PMNs at various cell-densities after fMLP-stimulation at time 0. ■, 6.4×10^5/cm²; ○, 1.6×10^5/cm²; ●, 0.2×10^5/cm². Each point represents the mean of the results from 2 wells. For each well, at least 3 different areas were counted.

TABLE 1
Effect of fixed PMNs and lymphocytes on superoxide generation by adherent PMNs[a]

	Control	+Fixed PMNs	+Lymphocytes
O_2^- generation (nmol/well, 15 min)	4.3±0.3	4.5±0.1	4.8±0.2

[a]Each well contained 3.2×10^5 live PMNs at a density of $3.2\times10^5/cm^2$. The cytochrome c reduction assay in 15 min induced by fMLP stimulation was conducted in the presence and the absence of fixed PMNs and lymphocytes (6.4×10^5/well). The results are expressed as mean ±s.d. of four experiments.

the reaction mixture of cytochrome c reduction assay at a lymphocytes: PMNs ratio of 2, but they showed no inhibition (Table 1).

Effect of a NO Synthetase Inhibitor and Adenosine Deaminase

NO has been reported to inhibit NADPH oxidase.[17] Adenosine which could be released from damaged PMNs is known to be a potent inhibitor of superoxide generation.[18] However, neither the pretreatment of PMNs with a NO synthetase inhibitor, monomethylarginine (200 μM), nor the addition of adenosine deaminase (0.25 U/ml), increased superoxide generation at high cell densities (Table 2). Superoxide generation at the higher cell density was further suppressed by monomethylarginine.

DISCUSSION

In this study, inhibition of superoxide generation at higher cell densities was demonstrated. Without changing the total cell number, cell area densities were altered by changing the distribution area of PMNs. In both cytochrome c reduction and spin trapping, the ratio of superoxide generation at 6.4×10^5 cells/cm^2 compared to at 3.2×10^5 cells/cm^2 was around 70% (Figures 2, 3). When the total cell number was changed without altering the distribution area, superoxide generation was saturated when cell numbers were large (Figures 4, 5, 6). The result with H_2O_2 generation was comparable to that of superoxide (Figure 7). Because the scale of the abscissa in Figures 4, 5, 6 and 7 is the same, the similarity of dose-response curves of adherent PMNs in these figures

TABLE 2
Effect of MMA[a] and ADA[b] on superoxide generation by adherent PMNs

	Control	MMA[d]	ADA[e]
O_2^- generation (nmol/well,[c] 15 min)			
A) cell density: $1.6\times10^5/cm^2$	18.0±1.1[f]	17.2±1.3	17.6±0.4
B) cell density: $6.4\times10^5/cm^2$	8.6±0.8	5.8±0.2[g]	9.0±0.4
B/A (%)	47.8	33.7	51.1

[a]MMA, monomethylarginine.
[b]ADA, adenosine deaminase.
[c]Each well contained 6.4×10^5 PMNs.
[d]After pretreatment of PMNs with 200 μM MMA, superoxide in the presence of MMA was determined.
[e]The reaction mixture contained 0.25 U/ml ADA.
[f]The results are expressed as mean ±s.d. of at least 3 experiments.
[g]$p<0.05$ v.s. control by Student t test.

strongly suggests that inhibition of the respiratory burst was cell-density dependent. The inhibition at higher cell densities was observed not only fMLP-stimulated cells but also PMA stimulated cells. This suggests that density-dependent inhibition was mediated through later phase of NADPH oxidase activation process than earlier signal transduction pathway which initiated by cell-membrane receptor-ligand coupling.

We hypothesized that inhibition by mutual cell contact of PMNs is the cause of reduced generation of superoxide. Microscopic observation of PMNs indicated that when the proportion of cells in mutual contact was less than 15% ($4 \times 10^4/cm^2$, Figure 1), rates of superoxide generation were proportional to the cell density (Figures 4, 5, 6). As the density increased, the ratio of cells with mutual contact increased (Figure 1), and inhibition of superoxide generation became apparent (Figures 4, 5, 6). In contrast to adherent cells, saturation was not observed when PMNs were in suspension, possibly because mutual contact may not easily occur when the cells are in suspension (Figures 4, 5).

Alternative explanations to the observed phenomenon are listed as follows: 1) Superoxide is generated equally at higher cell densities as at lower densities, but it becomes difficult for the assay probe (cytochrome c or DMPO) to access the generation site. 2) PMNs harm each other at higher densities, and as a result of decreased viability, they generate smaller amounts of superoxide. 3) Certain mediator(s) secreted by PMNs inhibit superoxide generation. These hypotheses were tested experimentally, and none appear to adequately explain inhibition of superoxide generation at high cell density. The first possibility appears unlikely from the results of H_2O_2 measurements. Superoxide may not easily escape from interfaces of membranes, because it has a negative charge. In contrast, H_2O_2 diffuses like water,[19] and reacts with HRP in the solution. The saturation pattern in H_2O_2 generation (Figure 7) was similar to that observed in superoxide assays (Figures 4, 5, 6), indicating that the respiratory burst itself was inhibited at higher densities of the cells. The second possibility was directly disproven by the observation that at higher densities, most PMNs survived after hours of incubation (Figure 8). This observation is consistent with the nature of PMNs as suicide attackers.[2] In addition, this observation also argues against the first possibility. If superoxide and related active species were not free to diffuse from their site of generation to react with molecular probes, they should react locally to enhance cell death. Further, this finding suggests that inhibition by mutual contact may be physiologically relevant at sites of inflammation. The third possibility is also unlikely because of the following reasons: The density-dependent inhibition of superoxide generation was observed even when the cell number per well, as well as per ml medium was kept constant (Figure 2). Under these conditions, the concentrations of long-lived mediators such as granular enzymes in the medium should be the same. More specifically, in this study, adenosine and NO were demonstrated not to be the causes of cell density-dependent inhibition of superoxide generation (Table 2). Further inhibition by monomethylarginine of superoxide generation at the higher cell density suggests that NO might have antagonized in the inhibition of superoxide generation by mutual contact. However, it is quite possible that other mediators are involved.

It is also unlikely that oxygen depletion in the EPR cuvette can explain inhibition of DMPO-OOH signals at high densities. The concentration of dissolved oxygen (about 60 nmol/200 μl) should be sufficient to maintain the respiratory burst for several minutes, even if adherent PMNs were capable of generating superoxide at rates observed with PMA-stimulated cells in suspension (Figure 5). In addition to that, the same phenomenon was observed in the Figures 4, 5 and 7, in which oxygen limitation was not a factor.

The molecular mechanism of the inhibition has not been elucidated. It is possible that cell-cell interaction through ligand(s)-receptor(s) coupling is involved. However, the absence of inhibition by fixed PMNs indicates that mere physical contact may not be enough, and that subsequent mutual interaction might be necessary to induce significant inhibition.

As we have discussed elsewhere,[9] discrepant results in the literature about the effect of adhesion on superoxide generation by PMNs could be due to the differences in cell densities. In reports which describes that adhesion inhibits the respiratory burst, the cell densities employed were higher than 0.6 million /cm^2.[4-6] In contrast, authors who reported positive effects of adhesion on the respiratory burst used much lower concentrations, such as 0.2–0.4 million/cm^2[7] or 0.05 million/cm^2.[8] When we used PMA as stimulant, PMNs in suspension generated more superoxide compared to adherent PMNs (Figure 5). The smaller generation in adherent cells may be due to inhibition by mutual contact of PMNs. In contrast, when fMLP was used as stimulant, adherent PMNs generated more superoxide than PMNs in suspension. This is consistent with the known phenomenon that adherent PMNs generate superoxide continuously for hours, while PMNs in suspension generate superoxide only for several minutes.[7-9] As a result of these factors, adherent PMNs generate more, or less, superoxide compared to PMNs in suspension, depending on the density and the stimulus (Figures 4, 5).

Acknowledgements

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