Na⁺/H⁺ Exchange Modulates Rat Neutrophil Mediated Tumor Cytotoxicity

Akemi Araki, Tomio Inoue, Edward J. Cragoe, Jr., and Fujiro Sendo

Department of Parasitology, Yamagata University School of Medicine, Yamagata 990-23, Japan [A. A., T. I., F. S.], and Nacogdoches, Texas 75963-1548 [E. J. C.]

ABSTRACT

Rat neutrophils stimulated with phorbol 12-myristate 13-acetate, Bacillus Calmette-Guérin, zymosan A, and β-1,3-d-glucan from Alcaligenes faecalis showed cytotoxicity to various tumor cells. Hydrogen peroxide was shown to be an effector molecule in tumor cytotoxicity by inhibition using various active oxygen scavengers. The following findings suggest that tumor cytotoxicity by rat neutrophils stimulated with the four reagents mentioned above is regulated by Na⁺/H⁺ exchange: (a) an increase in extracellular pH (pHₑ) from 6.5 to 8.0 resulted in enhancement of both tumor cytotoxicity and H₂O₂ production; (b) amiloride and its derivatives, inhibitors of Na⁺/H⁺ exchange, inhibited both functions of neutrophils mentioned above; (c) amiloride reduced intracellular pH (pHᵢ) of neutrophils stimulated with the four reagents; (d) a decrease in the extracellular concentration of Na⁺ ([Na⁺]ₑ) inhibited H₂O₂ production; (e) monensin, a Na⁺/H⁺ exchange ionophore, enhanced tumor cytotoxicity by neutrophils.

INTRODUCTION

Tumor cytotoxicity by human (1–3) and rodent neutrophils (4–6) has been demonstrated by many workers, and its effector molecules have been shown to be active oxygen species (7, 8) and cationic proteins (9, 10). However, the precise mechanisms of tumor cytotoxicity by neutrophils remain obscure.

Recently, Na⁺/H⁺ antiporter has been recognized as the main system in the regulation of pHᵢ in vertebrate cells, and extensive studies have been carried out using cells or tissues such as fibroblasts (11–14), HL-60 (15, 16), rat uterine smooth muscle (17), gastric glands (18), lymphocytes (19, 20), and erythrocytes (21). With regard to neutrophils, participation of Na⁺/H⁺ antiporter has been demonstrated in the regulation of pHᵢ in cells stimulated with PMA (22–26), N-formylmethionylleucylphenylalanine (27–32), leukotriene B₄ (33), or a combination of granulocyte-macrophage-colony-stimulating factor and N-formylmethionylleucylphenylalanine (34). Furthermore, a change in pHᵢ induced by Na⁺/H⁺ exchange may modulate neutrophil function such as O₂ (29) or leukotriene B₄ production (35) and chemotaxis or chemokinesis (32).

During our investigation, we discovered by chance that a change in the pH of the reaction medium resulted in an increase or decrease of tumor cytotoxicity by rat neutrophils. Through further study, it was revealed that tumor cytotoxicity by neutrophils treated with certain stimulators is modulated by Na⁺/H⁺ antiporter.

MATERIALS AND METHODS

Rats and Tumor Cells. WKA/Hkm rats were purchased from Shizuoka Animal Co., Ltd., Shizuoka, Japan. RLd1 cells (from an X-irradiation induced lymphoma of a BALB/c mouse) were used as target cells in cytology assays. They were passaged in tissue culture. (We also used other tumor cell lines as target cells in preliminary experiments and found that RLd1 was the most sensitive to neutrophil cytotoxicity. Accordingly, we used the RLd1 cell line as a source of target cells.)

Culture Media. IMDM (Gibco Laboratories, Chagrin Falls, OH) supplemented with 10% heat inactivated FCS (Gibco), 100 μg/ml streptomycin, 100 units/ml penicillin G, and 3 mg/ml NaHCO₃ was used for cytology assay, except when otherwise stated. To prepare a pH adjusted culture medium, the pH of IMDM supplemented with streptomycin, penicillin G, and NaHCO₃ in the doses described above was adjusted with 1 N HCl and 1 N NaOH. RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin G, 2 mg/ml NaHCO₃, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Digin Laboratory Co., Ltd., Kumamoto, Japan) was used for maintenance of RLd1 cells.

Preparation of Neutrophils. Twenty ml of 3% proteose peptone (Difco, Detroit, MI) were injected i.p. into WKA/Hkm rats, and an identical booster injection was administered 12 h later. At 2 to 3 h after the booster injection, peritoneal exudate cells were obtained by peritoneal lavage. Purification of neutrophils from peritoneal exudate cells was performed by centrifugation on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden), as described elsewhere (36). Neutrophils made up more than 98% of the final preparation, as determined by May-Giemsa staining.

Chemoattractants. The chemoattractants used were: zymosan A (Difco, Detroit, MI); BCG, Bacillus Calmette-Guérin (Difco, Detroit, MI); EIPA, 5-(A'-ethyl,-V-isopropyl)amiloride (Cayman Chemical, Ann Arbor, MI); and monensin, a Na⁺/H⁺ exchange ionophore. The preparation was performed by centrifugation on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden), as described elsewhere (36).

Cytolysis Assay. The cytolytic effect of neutrophils (10⁷) was determined by adding 10⁵ labeled target cells to each well in the 96-well plate. The plate was incubated at 37°C for 4 h, and the lysis was determined by a liquid scintillation counter (Rackbeta 1216; LKB Wallac, Turku, Finland). The percentage of specific lysis was calculated as

% lysis = \( \frac{cpm \text{ of experimental group} - cpm \text{ of spontaneous release}}{cpm \text{ of maximal release} - cpm \text{ of spontaneous release}} \times 100 \)

The maximum release was determined by substituting 1 N HCl for neutrophils, and spontaneous release was determined by culturing target cells alone. Assays were repeated at least three times, and representative data were collected.

Hydrogen Peroxide Assay. The method reported by Root et al. (37) was used. The reaction mixture consisted of scopoletin (2 to 4 μM final concentration), horseradish peroxidase (22 nM final concentration), and various neutrophil suspensions (2 × 10⁶) and stimulants, in the presence or absence of amiloride. The total volume of the reaction mixture was 3 ml. The test solution consisted of 135 mM NaCl, 5 mM KCl, 0.6 mM CaCl₂, 1 mM MgSO₄, 5 mM glucose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. pH was adjusted to 6.5.
to 8.0 by HCl and NaOH. After incubation for 30 min at 37°C, the fluorescence of the supernatants was measured with a spectrofluorometer (Hitachi 204-R) with excitation at 350 nm and emission at 460 nm. To test the effect of extracellular Na+, N-methyl-d-glucamine* solution was prepared by isosmotic replacement of NaCl by N-methyl-d-glucamine chloride.

Cytoplasmic pH (pH¡) Measurement. pH¡ was determined fluorometrically using BCECF, essentially as described by Grinstein and Furuya (27). The test solution was the same as used in the H2O2 assay. Rat neutrophils (3 x 10^6/ml) were loaded with the probe by incubation with the precursor BCECF acetoxymethyl ester (3.0 μM) for 30 min at 37°C with gentle stirring. The cells were then washed free of the extracellular probe and suspended at a concentration of 1 x 10^6 cells/ml. The mixtures of 5 x 10^6 neutrophils containing various neutrophil stimulants were incubated for 30 min at 37°C in the presence or absence of amiloride in a total volume of 3 ml, after adjusting pH¡ to 6.5, 7.4, or 8.0. After incubation, the fluorescence of the supernatants was measured with a spectrofluorometer with emission and excitation wavelengths of 500 and 530 nm, respectively. The nigericin K* method of Thomas et al. (38) was used to calibrate pH¡.

Reagents. PMA, Zy A, monensin, ouabain, amiloride, N-methyl-d-glucamine chloride, scopolamine, and horseradish peroxidase were purchased from Sigma Chemical Co. HMA and EIPA were synthesized as described previously (39, 40). BCG was purchased from Nippon BCG Co. (Tokyo, Japan). Linear β-1,3-d-glucan isolated from Alcaligenes faecalis var. IFO 13140 (TAK) was kindly supplied by Takeda Pharmaceutical Co. (Osaka, Japan). BCECF acetoxymethyl ester was obtained from Dojindo Laboratories, Kumamoto, Japan.

Statistical Studies. Statistical analyses were performed using Student's t test.

RESULTS

Effect of pH¡ on Tumor Cytotoxicity by Neutrophils Stimulated with PMA, BCG, Zy A, or TAK. It has been demonstrated previously that mouse neutrophils stimulated with various reagents such as PMA, BCG, Zy A, and TAK show significant cytotoxicity to various tumor cells (4, 7, 36). First, we examined whether this is true in the case of rat neutrophils. As shown in Table 1, rat neutrophils showed significant tumor cytotoxicity when treated with PMA, BCG, Zy A, and TAK, although the average intensity of cytotoxicity was lower than that of mouse neutrophils. We then examined the effect of scavengers of active oxygen on tumor cytotoxicity by rat neutrophils to identify the effector molecules. Of the scavengers used (catalase, Superoxide dismutase, histidine, and mannitol), only catalase completely inhibited cytotoxicity (Fig. 1), which suggests that H2O2 is an effector molecule in this system as in cytotoxicity by mouse neutrophils (7, 36).

The above experiments were performed at a pH¡ of 7.2–7.4, the pH range of the usual culture media. We next changed the pH¡ from 6.5 to 8.0 in order to observe the effect on tumor cytotoxicity. As shown in Fig. 2, cytotoxicity by neutrophils was stimulated with the four reagents and increased relative to the pH¡ value. On the other hand, a change in pH¡ within the range of 6.5 to 8.0 did not affect tumor cytotoxicity of nonstimulated neutrophils, which was approximately 5%. Spontaneous release of [3H]deoxyuridine from target cells alone did not change at a pH¡ of 6.5 to 8.0, although at values higher or lower than this range spontaneous release was rather high. Taking this into consideration, further experiments were performed at pH¡ 6.5 to 8.0.

Effect of pH¡ on H2O2 Production by Stimulated Neutrophils. We demonstrated that one effector molecule in this system may be H2O2, and we also showed that cytotoxicity is regulated by pH¡. We then decided to study the effect of pH¡ on H2O2 production by stimulated neutrophils. Fig. 3 shows that the amount of H2O2 produced by neutrophils treated with the four stimulants increased relative to the pH¡ value. Neutrophils stimulated with PMA, in particular, showed a sharp increase in H2O2 production. The stability of H2O2 was not affected by a change at a pH¡ of 6.5 to 8.0. Neutrophils treated with PMA, BCG, Zy A, and TAK, when the pH¡ value was increased from 6.5 to 8.0, showed a 3-fold increase in H2O2 production. The stability of H2O2 was not affected by a change in pH¡ within the range of 6.5 to 8.0.

Effect of [Na+]o, on H2O2 Production by Stimulated Neutrophils. Preparing a culture medium suitable for examining the effect of [Na+]o on tumor cytotoxicity by neutrophils is ex-

Table 1 Tumor cytotoxicity by rat neutrophils stimulated with PMA, BCG, Zy A, and TAK

<table>
<thead>
<tr>
<th>Neutrophil stimulant</th>
<th>% of specific lysis (E/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>PMA</td>
<td>27.1</td>
</tr>
<tr>
<td>BCG</td>
<td>19.5</td>
</tr>
<tr>
<td>Zy A</td>
<td>11.0</td>
</tr>
<tr>
<td>TAK</td>
<td>10.3</td>
</tr>
</tbody>
</table>

* PMA, 100 ng/ml; BCG, Zy A, TAK, 500 μg/ml. Incubation time, 24 h; pH¡, 7.2. Nonstimulated neutrophils showed less than 5% tumor cytotoxicity. Spontaneous release (%): 18.4 ± 0.3 (Experiment 1); 21.4 ± 0.1 (Experiment 2). E/T, effector/target ratio.
Na+/H+ Exchange Modulates Tumor Cytotoxicity

Fig. 3. Effect of pH0 on H2O2 production by neutrophils stimulated with PMA, BCG, Zy A, and TAK. PMA, 100 ng/ml; BCG, Zy A, TAK, 500 
5g/ml. pH0 7.4. Nonstimulated neutrophils produced less than 0.5 nmol/10^6 cells of H2O2.

Effect of pH0 on H2O2 production by neutrophils stimulated with PMA, BCG, Zy A, and TAK. PMA, 100 ng/ml; BCG, Zy A, TAK, 500 
5g/ml. pH0 7.4. Nonstimulated neutrophils produced less than 0.5 nmol/10^6 cells of H2O2.

Effect of Amiloride and Its Derivatives on Tumor Cytotoxicity and H2O2 Production by Stimulated Neutrophils.

Reduction of H2O2 production by stimulated neutrophils was observed when the [Na+]o concentration was gradually lowered (Fig. 4), which shows that Na+ is required for production of H2O2. Furthermore, when neutrophils in the [Na+]o deficient solution were transferred to the standard test solution containing Na+, H2O2 production was restored completely, indicating that the decreased H2O2 production in the low [Na+]o solution could not be caused by a toxic effect of N-methylglucamine.

Effect of Amiloride and Its Derivatives on Tumor Cytotoxicity and H2O2 Production by Stimulated Neutrophils. We next examined the effect of amiloride and its derivatives, inhibitors of Na+/H+ exchange, on tumor cytotoxicity and H2O2 production by neutrophils stimulated with the four reagents. Cytotoxicity by PMA stimulated neutrophils was inhibited by amiloride and its derivatives in a concentration dependent manner (Fig. 5). Similar inhibition of tumor cytotoxicity by amiloride, HMA, or EIPA was also observed in neutrophils stimulated with BCG, Zy A, or TAK (data not shown). The order of inhibition of cytotoxicity and H2O2 production was the same as that of inhibition of Na+/H+ exchange by these reagents (87). Amiloride and its derivatives, at the concentrations used, showed no effect on viability of target tumor cells and neutrophils. The same finding was obtained with H2O2 production (Fig. 6). These results suggest that H2O2 mediated tumor cytolysis by neutrophils stimulated with the four reagents is modulated by an amiloride sensitive Na+/H+ exchange.

Change of pHi of Stimulated Neutrophils. Next, we examined the pHi of neutrophils stimulated with the four agents. The results were: (a) the increase in the intracellular pH of stimulated or nonstimulated neutrophils paralleled the increase in

Table 2. Effect of pHi and amiloride on pHi change in stimulated neutrophils.

Details of pHi measurement are given in “Materials and Methods.”

| Amiloride (100 

<table>
<thead>
<tr>
<th>pH0</th>
<th>pHi</th>
<th>pH0</th>
<th>pHi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonstimulated</td>
<td>7.20 ± 0.02</td>
<td>7.32 ± 0.01</td>
<td>7.66 ± 0.01</td>
</tr>
<tr>
<td>PMA (100 ng/ml)</td>
<td>6.82 ± 0.03</td>
<td>6.90 ± 0.02</td>
<td>7.20 ± 0.04</td>
</tr>
</tbody>
</table>
| BCG (500 

<table>
<thead>
<tr>
<th>pH0</th>
<th>pHi</th>
<th>pH0</th>
<th>pHi</th>
</tr>
</thead>
</table>
| Zy A (500 

<table>
<thead>
<tr>
<th>pH0</th>
<th>pHi</th>
<th>pH0</th>
<th>pHi</th>
</tr>
</thead>
</table>
| TAK (500 

* Mean ± SD.
* P < 0.01, compared with amiloride negative control groups.

3214
pH₃ (see Table 2); (b) when neutrophils were stimulated with PMA, pH decreased compared with that of nonstimulated neutrophils; (c) the intracellular pH of neutrophils stimulated with BCG, Zy A, or TAK did not change compared with that of nonstimulated control neutrophils; (d) addition of amiloride reduced the pH₃ of neutrophils stimulated with the four reagents, while no change in pH was observed when nonstimulated neutrophils were treated with amiloride. For example, when the pH₃ was 7.4, pH₃s of neutrophils stimulated with BCG, Zy A, and TAK in the absence of amiloride were 7.32 ± 0.05 (SD), 7.32 ± 0.01, and 7.38 ± 0.01, respectively, and they changed to 7.08 ± 0.02, 7.00 ± 0.02, and 7.18 ± 0.01, respectively, in the presence of 100 μg/ml amiloride. On the other hand, pH₃s of nonstimulated neutrophils under these conditions were 7.32 ± 0.01 and 7.35 ± 0.06 in the absence or presence of amiloride, respectively.

Effect of Monensin on Tumor Cytotoxicity and H₂O₂ Production by Neutrophils. We next observed the effect of a Na⁺/H⁺ exchanging ionophore, monensin, on tumor cytotoxicity by neutrophils. Monensin augmented tumor cytotoxicity in a dose dependent manner. The augmentation rate was higher in untreated neutrophils than in PMA stimulated ones (Fig. 7). Similar augmentation of tumor cytotoxicity by monensin was also observed in neutrophils stimulated with BCG, Zy A, or TAK (data not shown). As for H₂O₂ production, monensin showed a slight but significant augmenting effect in both PMA stimulated and nonstimulated neutrophils (data not shown). A blocker of Na⁺/K⁺ exchange, ouabain, had no effect on either tumor cytotoxicity or H₂O₂ production, which suggests that Na⁺/K⁺ exchange is not involved in this system.

DISCUSSION

We demonstrate in this paper that H₂O₂ mediated tumor cytotoxicity by rat neutrophils stimulated with certain reagents is modulated by Na⁺/H⁺ antipporter. This conclusion is supported by the following results: (a) tumor cytotoxicity and H₂O₂ production was enhanced with an increase in pH₃; (b) H₂O₂ production increased relative to the concentration of [Na⁺]₀; (c) tumor cytotoxicity and H₂O₂ production was inhibited by blockers of Na⁺/H⁺ exchange; (d) a Na⁺/H⁺ exchange ionophore enhanced tumor cytotoxicity. The roles of other pH₃ control systems, such as the bicarbonate transport system, in modulating of neutrophil tumor cytotoxicity remain to be clarified.

The mechanisms of Na⁺/H⁺ antipporter involvement in modulation of tumor cytotoxicity by neutrophils are still poorly understood, as are mechanisms concerned with the modulation of other neutrophil functions (29, 32, 35). We do know, however, that certain point(s) in the pathway for H₂O₂ production or release may be affected by Na⁺/H⁺ antipporter, inasmuch as one effector molecule in tumor cytotoxicity was speculated to be H₂O₂. Furthermore, it has been demonstrated by Nakagawa et al. (42) that H₂O₂ production by human monocytes stimulated with PMA is enhanced by increasing pH₃.

Tumor cytotoxicity and H₂O₂ production by rat neutrophils was lowered by inhibitors of Na⁺/H⁺ exchange in much smaller concentrations than those producing effective inhibition of various human neutrophil functions (Figs. 5 and 6) (24, 29). Furthermore, the order of the inhibition potential of amiloride and its derivatives paralleled that of Na⁺/H⁺ exchange inhibition which was reported previously (35). Concentrations causing half-maximal inhibition of tumor cytotoxicity by amiloride, HMA, and EIPA were 30, 1, and 1 μM respectively. These results suggest that (a) Na⁺/H⁺ exchange in rat neutrophils may be much weaker than human neutrophils and that (b) inhibition of rat neutrophil functions by amiloride and its derivatives is not caused by side functions such as a Na⁺ channel block but by the Na⁺/H⁺ exchange block itself.

As for the change in pH₃ of stimulated neutrophils in this study, a more lengthy discussion is required. At a physiological pH₃, an initial cytoplasmic acidification followed by an alkalinizing phase (22-26, 27, 28, 33) or alkalinization alone (43) has been reported using stimulated human neutrophils. In contrast with these previous results, at a physiological pH₃, we observed cytoplasmic acidification (in the case of PMA stimulation) or a lack of change in pH₃ (with BCG, Zy A, and TAK stimulation) in stimulated rat neutrophils. These discrepancies may be reconciled by the following. Na⁺/H⁺ antipporter in peritoneal rat neutrophils is likely much less potent compared with that of peripheral blood human neutrophils, inasmuch as tumor cytotoxicity and H₂O₂ production were inhibited with Na⁺/H⁺ exchange inhibitors at much smaller concentrations than those required for inhibition of various functions of human neutrophils. As a result, cytoplasmic acidification by PMA which might be induced by accumulation of H⁺ liberated during the metabolic burst (24-26) was not restored even by Na⁺/H⁺ antipporter. The lack of an apparent change in pH₃ of rat neutrophils stimulated with BCG, Zy A, and TAK may reflect weak H⁺ liberation during the metabolic burst and a weak Na⁺/H⁺ antipporter in these neutrophils. An involvement of a weak but significant Na⁺/H⁺ antipporter in rat neutrophils stimulated with PMA, BCG, Zy A, or TAK is suggested by the fact that the pH₃ of these neutrophils stimulated with the four reagents was reduced through treatment with amiloride, while the pH₃ of nonstimulated neutrophils did not change with this treatment.

We have already demonstrated that neutrophils stimulated with various cytokines show tumor cytotoxicity (44-46). In contrast to our observations in the present study using PMA, BCG, Zy A, and TAK, tumor cytotoxicity induced by cytokines was little affected by a change in pH₃. These results suggest that tumor cytotoxicity regulated by Na⁺/H⁺ antipporter is dependent on neutrophil stimulators and cytotoxic effector molecules, inasmuch as we already demonstrated that the effector molecule(s) in cytokine induced neutrophil cytotoxicity may not be active oxygens but certain cationic protein(s) (9).
thermore, tumor cytotoxicity of mouse neutrophils stimulated with the four reagents used in this study was less affected by Na+/H+ antiporter. 3 Thus, the degree of modulation of neutrophil cytotoxicity by Na+/H+ exchange is dependent on species.

REFERENCES

Na\(^+\)/H\(^+\) Exchange Modulates Rat Neutrophil Mediated Tumor Cytotoxicity

Akemi Araki, Tomio Inoue, Edward J. Cragoe, Jr., et al.