Production of Factor(s) That Render Polymorphonuclear Leukocytes Cytotoxic from Spleen Cells Stimulated with a Streptococcal Preparation, OK-432

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ABSTRACT

We previously reported the augmentation of tumor cytotoxicity of polymorphonuclear leukocytes (PMN) by in vivo administration of a streptococcal preparation, OK-432 (S. Watabe et al., J. Natl. Cancer Inst., 72: 1365–1370, 1984). The present study was undertaken to elucidate the mechanisms of the phenomena. Mouse and rat spleen cells were stimulated in vitro with OK-432. The culture supernatants from the stimulated spleen cells (OK sup) contained factor(s) that rendered mouse and rat PMN cytostatic [neutrophil activating factor (NAF)]. The stimulation of spleen cells with a small dose of OK-432 (0.05 μg/ml) resulted in the production of maximum NAF, and NAF was produced soon (12 h) after OK-432 stimulation. NAF was partially inactivated with 60°C for 30 min, and completely inactivated with 100°C for 10 min. NAF was sensitive to pH 2 treatment. The treatment of PMN with OK sup for 5 min at 37°C was sufficient to induce cytostatic activity of PMN. That OK sup contained γ-interferon and recombinant γ-interferon showed NAF activity indicate that γ-interferon is a NAF in OK sup.

INTRODUCTION

The effector mechanisms in host-tumor relationships have been studied by many investigators, and considerable information about effector cells in the host defense against cancer has been obtained. Of these effector cells, PMN have attracted relatively little attention (1–9). However, recent studies demonstrated that PMN might play an important in vivo role in rejecting tumor cells, especially in the hosts treated with certain bacterial preparations (10, 11–15). In a previous paper, we demonstrated that the injection of a streptococcal preparation, OK-432, into the peritoneal cavity of rats induced PMN which showed cytotoxicity against tumor cells (10). In regard to the mechanisms of induction of cytotoxic neutrophils by in vivo administration of OK-432, neutrophil activation may be induced by the direct effect of OK-432 on neutrophils. We showed in the previous paper that in vitro incubation of neutrophil suspensions with OK-432 induced cytotoxicity of neutrophils (10). Also, neutrophil activation induced by in vivo administration of OK-432 may occur through certain lymphokines produced by lymphoid cells stimulated with OK-432. Saito et al. have demonstrated that mouse spleen cells stimulated with OK-432 produce γ-interferon (16). And from our previous result (17), cytotoxicity of PMN can be augmented by certain lymphokines. Furthermore, it has been reported that γ-interferon and tumor necrosis factor activate various kinds of neutrophil functions (18, 19). The present study was undertaken to investigate whether the stimulation of lymphoid cells by a bacterial preparation, OK-432, induces some factors which render PMN cytotoxic. In this paper, we demonstrate that rat and mouse spleen cells stimulated with OK-432 produce factors that augment PMN cytotoxicity. In this paper we use "cytotoxicity" in a broad sense to include both cytostasis and cytolysis. We tentatively named the factors produced from lymphoid cells which render neutrophil cytotoxic, "neutrophil activating factor(s)."

MATERIALS AND METHODS

Animals and Tumor Cells

Donryu SPF rats, and BALB/c and C3H/HeN mice were purchased from Shizuoka Laboratory Animal Co., Ltd., Shizuoka, Japan. Wistar King Aptekamen/Hok (WKA/Hok) rats were raised in our laboratory colony. Tumor cell lines used as target cells in the cytotoxicity assay were RL1 (BALB/c mouse lymphoma), Meth A (BALB/c fibrosarcoma), KMT-17 (WKA rat fibrosarcoma), and AH-66 (Donryu rat liver cell carcinoma).

Preparation of PMN Suspensions

The detail of obtaining rat PMN was described in the previous paper (10). The purity of obtained neutrophil suspensions was more than 99% as determined by May-Giemsa staining. Preparation of mouse peritoneal PMN followed the method reported by Hansen et al. (20) and Goto et al. (21). BALB/c mice were i.p. injected with 2 ml of 3% proteose peptone twice at 12 and 3 h before the cell harvest. The obtained peritoneal exudate cells were placed on a discontinuous gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), and centrifuged at 400 × g for 1 h at room temperature. Percoll gradient consisted of 70, 65, 60, 50, and 30% purity. The obtained cell suspensions at the bottom layer consisted of 98.6% PMN, 0% lymphocytes, and 1.4% macrophages.

Supernatants of Spleen Cells Stimulated with OK-432

Varying numbers of spleen cells from BALB/c mice and WKA rats were incubated at 37°C under 5% CO2 and 95% air in the presence or absence of a streptococcal preparation, OK-432 (22), supplied by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. One mg of OK-432 is equivalent to 10KE units used by some other workers. The culture medium consisted of RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Flow Laboratories, Inc., Rockville, MD). At the end of incubation, culture supernatants were obtained by centrifugation of culture cell suspensions at 1,700 × g for 30 min. Supernatants of culture of OK-432 in the absence of spleen cells were also used as a control after being centrifuged. The supernatants were passed through an HA Millipore filter (Millipore, Japan, Co., Ltd., Tokyo, Japan) and were stored at −40°C until used.

Treatment of PMN by Supernatants from Spleen Cells Stimulated with OK-432 (OK sup)

One hundred μl of varying concentrations of PMN were incubated with the same amount of OK sup at 37°C in a Falcon Micro Test Plate II 3042 (Falcon Plastics, Los Angeles, CA). After the incubation, the treated PMN were washed three times with minimum essential medium (Nissui, Tokyo, Japan).
Cytotoxicity Assay

Cytostasis Assay. The details were described in the previous report (10). The percentage of inhibition was calculated as follows:

\[ \text{% Inhibition} = \left( 1 - \frac{\text{cpm of experimental group}}{\text{cpm of nontreated PMN}} \right) \times 100 \]  (A)

Cytolysis Assay. The details were described in the previous paper (10). Percentage of specific lysis was calculated as follows:

\[ \text{% Specific lysis} = \left( \frac{\text{cpm of experimental group}}{\text{total releasable cpm}} - \frac{\text{cpm of spontaneous release}}{\text{total releasable cpm}} \right) \times 100 \]  (B)

All culture wares, media, and sera used for in vitro examination were endotoxin-free as determined by Limulus assay.

Other Cytokine Assays

The details were described in the previous paper (23). Briefly, each cytokine assay was as follows.

Interleukin-1. The method reported by Conlon et al. was used (24). C3H/HeN mouse thymocytes (5 x 10⁶/well), which had not been agglutinated with peanut agglutinin, were seeded to a microplate, and a test sample was added to each well. The reaction mixtures were incubated in a CO₂ incubator for 72 h. The supernatants of 1 x 10⁶/ml adherent peritoneal exudate cells from Donryu rats stimulated with silica particles (100 µg/ml; Sigma, SS05) were used as a standard IL-1 source in a final 1/4 dilution. The IL-1 unit was calculated as the reciprocal of the dilution of the sample corresponding to the half maximum cpm x 100.

Interleukin-2. IL-2 activity was assayed by measuring tritiated thymidine incorporation of cloned cytotoxic T-cells. Four thousand cloned cytotoxic cells were seeded in 100 µl of RPMI 1640 medium containing 2% FCS in a microplate together with 100 µl of serially diluted assay samples. The reaction mixtures were incubated for 20 h in a CO₂ incubator. Recombinant human IL-2 (a gift from Dr. J. Hamuro, Ajinomoto Research Institute, Yokohama, Japan) was used as a standard IL-2 source (100 U/ml). The IL-2 unit was calculated as the reciprocal of the dilution of the sample corresponding to the half maximum cpm x 100.

Interferon. IFN assays were carried out by microtitration of cytokine assay (25). Murine recombinant IFN-γ supplied by Toray Research Institute, Kamakura, Japan, was used as a standard IFN-γ source.

Colony Stimulating Factor. Bone marrow cells of C3/HeJ mice were cultured in 35-mm plastic petri dishes. To each dish was added 1 x 10⁶ bone marrow cells in 0.88% methyl cellulose (Sigma) in 1 ml of α-medium (Flow Laboratories Inc., Rockville, MD) containing 20% FCS and 20% test materials. Dishes were incubated for 7 days at 37°C in a CO₂ incubator. A standard CSF source was obtained from cultivation of BALB/c spleen cells for 7 days with 1 µg/ml pokeweed mitogen (Hohnen Oil Co. Ltd., Tokyo, Japan). A standard CSF was used in a final ¼ dilution. One unit of CSF was determined as the CSF amount increasing one colony/1 x 10⁶ bone marrow cells.

Statistical Methods

Statistical analyses were performed with Student's t test.

RESULTS

Production of Factor(s) That Render PMN Cytotoxic by Spleen Cells Incubated with OK-432. Five million spleen cells per ml of BALB/c mice and WKA rats were incubated for 48 h in the presence or absence of 5 µg/ml OK-432. As a control only OK-432 alone was incubated with the culture medium. At the end of the incubation, supernatants were obtained from each experimental group. Varying concentrations of rat or mouse PMN were mixed with each undiluted supernatant, and the reaction mixtures were incubated for 6 h at 37°C. After the removal of the supernatants by washing, RL21 cells were added to the treated PMN and cytostasis of the PMN against target cells was observed. Fig. 1 shows a typical result indicating that (a) Donryu rat PMN stimulated with OK sup from BALB/c spleen cells inhibits [³H]dThd uptake of the target cells, while supernatants from BALB/c spleen cells incubated in the absence of OK-432 barely enhanced cytostasis of rat PMN; (b) WKA spleen cells produce factors which to some extent augment the cytostasis of Donryu rat PMN even in the absence of OK-432, (the reason is unknown) but stimulation of WKA spleen cells with OK-432 significantly enhances the activity of the spleen cells to augment PMN cytostasis; and (c) supernatants from the culture of OK-432 itself show no effects on PMN cytostasis. OK sup-treated rat PMN showed cytostasis to various target tumor cells other than RL21 (data not shown). OK sup from various strains of rats and mice augmented cytostasis of PMN from not only rats but mice (data not shown). In the following experiments, we mainly used PMN from Donryu rats, OK sup from BALB/c mice, and RL21 as target tumor cells. We will tentatively call the factor(s) that render PMN cytotoxic neutrophil activating factor. In order to find an optimal condition for the production of NAF, varying concentrations of BALB/c spleen cells were incubated with OK-432 (5 µg/ml) for 48 h, and the NAF activity of each supernatant was examined. The incubation of 5 x 10⁶/ml and 1 x 10⁶/ml spleen cells with OK-432 gave the most active supernatants (data not shown).

We next examined the dose of OK-432 required for the production of NAF. Fig. 2 shows that BALB/c spleen cells stimulated with more than 0.05 µg/ml OK-432 produced maximum NAF, which indicates that a relatively small dose of OK-432 was required for the production of NAF. To test the kinetics of NAF production by spleen cells stimulated with OK-432, the supernatants were harvested at different times during incubation, and the NAF activity of each supernatant was examined. As shown in Fig. 3, NAF activity was not detected until 6 h,
Production of factor(s) that render PMN cytostatic

Table 1: Stability with physicochemical treatments of NAF in OK sup

<table>
<thead>
<tr>
<th>Treatment of OK sup*</th>
<th>$\frac{1}{2}$</th>
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<td>97 ± 3</td>
<td>93 ± 5</td>
<td>80 ± 6</td>
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<td>56°C 30 min</td>
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<td>75 ± 3</td>
<td>54 ± 2</td>
<td>49 ± 1</td>
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<tr>
<td>60°C 30 min$^d$</td>
<td>41 ± 6</td>
<td>24 ± 7</td>
<td>19 ± 3</td>
<td>9 ± 4</td>
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<tr>
<td>100°C 10 min$^e$</td>
<td>1 ± 5</td>
<td>3 ± 2</td>
<td>8 ± 1</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>16 ± 2</td>
<td>8 ± 2</td>
<td>9 ± 3</td>
<td>7 ± 3</td>
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<td>89 ± 1</td>
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<tr>
<td>Freezing and thawing</td>
<td>98 ± 0</td>
<td>95 ± 2</td>
<td>90 ± 2</td>
<td>79 ± 4</td>
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</table>

* E:T = 50:1.
$^d$ OK sup was obtained from BALB/c spleen cells incubated with 5 µg/ml OK-432 for 48 h at 37°C. Donryu rat PMN were treated with OK sup for 3 h at 37°C. E:T, mean ± SD, $^e$ Mean ± SD.

Fig. 2. Dose response of cytostasis by OK sup-treated PMN, to varying doses of OK-432 used for the stimulation of spleen cells. Five million per ml BALB/c spleen cells were incubated with varying doses of OK-432 for 48 h at 37°C. Donryu rat PMN was treated with the obtained OK sup for 3 h at 37°C. Effector-to-target ratio (E:T) = 50:1. Bars, mean ± SD of five experiments.

Fig. 3. Kinetics of production of NAF by spleen cells stimulated with OK-432. Five million per ml BALB/c spleen cells were incubated with 5 µg/ml OK-432 at 37°C for varying periods of the time. Donryu rat PMN was treated with the obtained OK sup for 3 h at 37°C. Effector-to-target ratio (E:T) = 50:1. Bars, mean ± SD of five experiments.

Fig. 4. Kinetics of activation of cytostatic PMN by OK-432. OK sup was obtained from BALB/c spleen cells stimulated with 5 µg/ml OK-432 for 48 h at 37°C. Donryu rat PMN were treated with OK sup for 3 h at 37°C. E:T = 50:1. Bars, mean ± SD of five experiments.

and first noticed after 12 h of incubation, and at this point it had already reached a plateau, which indicates that NAF was produced relatively soon after stimulation by OK-432.

Stability of NAF to Physicochemical Treatments. OK sup was treated at various temperatures, and the remaining NAF activity was examined. NAF was relatively resistant to the treatment at 56°C for 30 min, but the 60°C 30-min treatment partially abrogated NAF activity, and no significant NAF activity was detected after the 100°C 10-min treatment. Freezing and thawing did not affect the NAF activity of OK sup. Overnight dialization of OK sup with an RPMI medium at pH 2 cancelled the NAF activity, but dialization at pH 7 did not affect the NAF activity of OK sup (Table 1). These results indicate that NAF in OK sup was relatively unstable to physicochemical treatments.

Characteristics of PMN Cytotoxicity Augmented by OK sup. To examine the kinetics of PMN activation by OK sup, rat PMN were treated with OK sup at 37°C for varying periods of time, and the cytostatic activity of the treated PMN was examined. As shown in Fig. 4, a treatment of PMN with OK sup for 5 min had already induced the cytostatic activity of PMN, and with a 30-min treatment the maximum PMN activity was obtained. To examine the dose response of the PMN cytostasis augmented by OK sup, PMN were treated with serially diluted supernatants, and the treated PMN were tested for cytostatic activity at various effector-to-target ratios. The cytostasis by the treated PMN was dependent on the concentration of supernatants and on that of PMN (data not shown). Next the kinetics of cytostasis and cytolysis by the OK sup-treated PMN were observed. A significant inhibition of tritiated thymidine uptake by target cells appeared from 6 to 12 h after beginning the incubation of the target cells with the OK sup-treated PMN, and cytostasis gradually increased up to 24 h of incubation (Fig. 5A). On the other hand, a significant augmentation of cytolysis was first observed after 24 h of incubation (Fig. 5B). The cytolytic activity of rat PMN stimulated with mouse and rat OK sup was compared. As shown in Table 2, PMN stimulating activity of OK sup obtained from rat spleen cell culture was significantly stronger than that of OK sup obtained from the mouse, being the same results as in the cytostasis assay shown in Fig. 1. Supernatants from normal rat spleen cell culture stimulated rat PMN cytolysis to some extent especially when a
high effector-to-target ratio was employed. To determine relative species specificity between spleen cells producing NAF and PMN that are triggered, PMN from mice and rats were stimulated with OK-432 or medium for 12 h at 37°C. As shown in Table 3, OK sup contained IL-2 and IFN as already reported by Saito et al. (16), while OK sup did not show significant IL-1 and CSF activity. This result suggests that NAF in OK sup might be neither IL-1 nor CSF. Secondly, we examined NAF activity of recombinant IL-2 and IFN-γ. The condition of PMN preparation with these reagents was the same as that used in the case of OK sup. IFN-γ exhibited NAF activity. The kinetics of cytotoxic PMN generation and the actual kinetics of cytostasis and cytolysis induced were identical to those observed in the experiments performed using OK sup. However, IL-2 did not show any significant NAF activity (data not shown).

**DISCUSSION**

The present paper demonstrates that spleen cells stimulated with a streptococcal preparation, OK-432, produce factor(s) that render PMN cytostatic. We tentatively named this factor neutrophil activating factor. To our knowledge, this is the first report demonstrating that spleen cells stimulated with bacterial preparations produce factor(s) that render PMN cytostatic. Although the mechanisms of the induction of cytotoxic PMN by *in vivo* administration of OK-432 are far from clear, NAF production by lymphoid cells stimulated with this streptococcal preparation might be partially responsible for the *in vivo* phenomena. This assumption may not be too unreasonable for the following reasons: (a) the dose of OK-432 required for the induction of a significant NAF activity by spleen cells was far less than that of OK-432 required for the induction of cytostatic PMN through *in vitro* stimulation of PMN by OK-432, and (b) NAF was produced by spleen cells with as little as 12 h of incubation after OK-432 stimulation, which coincides with the previous observation that cytotoxic PMN appeared from 6 to 12 h after *in vivo* injection of OK-432. The present experimental results also suggest a general importance of certain lymphokines for the *in vivo* activation of the PMN function by microorganisms. The results supporting such an assumption have been reported in studies concerning the killing by PMN of bacteria (25), fungus (26), and amoeba (27). As for the reason why supernatants of rat spleen cells incubated in the absence of OK-432 rendered PMN cytostatic to some extents (Fig. 1), normal rat spleen cells might spontaneously produce a kind of NAF. Peritoneal macrophages from WKA rats used in the present experiments spontaneously produce IL-1 to some extent without any stimulation. As to the mechanisms of rapid activation of PMN by OK sup, two possibilities are conceivable: (a) PMN might have been primed in vivo by a minute amount of lipopolysaccharide, although we used SPF rats; (b) the time we showed as required for PMN activation might express that...
required for binding of NAF to its receptors on PMN, and longer time might be required for the actual activation of PMN function.

The physicochemical treatments of OK sup revealed that NAF produced by spleen cells stimulated with OK-432 was relatively unstable under such treatments. This result is different from the previous observation that NAF obtained from culture supernatants of rat spleen cells stimulated with concanavalin A was resistant to such treatments (17). These two results suggest that NAF consists of heterogeneous substances. Inasmuch as OK sup may contain various kinds of lymphokine, NAF must be purified before determining whether NAF in OK sup is a novel lymphokine or NAF activity is a function of other known lymphokines. Actually, it was recently reported that a purified colony stimulating factor has NAF activity (28). As shown in Table 2, OK sup did not show CSF activity, and our recent results indicated that CSF itself did not activate neutrophils in terms of augmentation of cytostatic activity (21). These two results suggest that NAF in OK sup was not CSF. Both of IL-1 and IL-2 may not be NAF in OK sup from the results shown in Table 2. Physicochemical stability of NAF in OK sup in temperature and pH treatment suggests that IFN-α/β may not be responsible for the phenomena observed in the present study. As to the relationships of NAF in OK sup with IFN-γ, we have not yet obtained a clear answer: (a) Mouse spleen cells stimulated with OK-432 produce IFN-γ (16); (b) NAF in OK sup was unstable to temperature and pH treatment as IFN-γ is; and (c) recombinant IFN-γ augmented cytostasis of neutrophils obtained from rats and mice. (These results cannot be ascribed to contaminated macrophages from the calculation based on the percentage of contaminated macrophages in neutrophil suspension and augmentation of macrophages cytostasis by IFN-γ). The above-mentioned observations strongly suggest that at least one of NAF in OK sup may be IFN-γ. As to whether NAF in OK sup is IFN-γ itself or certain other factor(s) are also responsible for NAF activity, the following results suggest that the latter is the case: (a) Anti-IFN-γ antibody completely abrogated NAF activity of recombinant IFN-γ, but it partially reduced NAF activity of OK sup; (b) linear regression analysis (29) between cytostasis activity and the IFN titer of OK sup showed that the line of cytostasis activity was more sharply rising, compared with that of the IFN titer; (c) when each fraction of gel filtration of OK sup in a process of IFN-γ purification was tested for its NAF activity, even in fractions which had no interferon activity, NAF activity was detected. Furthermore, it has been recently reported that OK-432 induces tumor necrosis factor (30), natural killer soluble cytotoxic factor, and monocyte cytotoxic factor (31) production, and that TNF activates PMN in terms of augmentation of antibody-dependent cell-mediated cytotoxicity (18). Thus, although a significant amount of lymphokinin was not detected in OK sup,4 TNF might be involved in the activation of PMN by OK sup, inasmuch as a very low dose of TNF can activate PMN. Furthermore, our preliminary experiments showed that recombinant TNF exhibited NAF activity even in the absence of antibody. Taken together, NAF in OK sup may consist of heterogeneous molecules. Actually, in our previous work, NAF activity of culture supernatants from rat spleen cells stimulated with concanavalin A was eluted in broad fractions by ion-exchange chromatography and gel filtration (23). More detailed physicochemical characterization of NAF in OK sup are under way through the purification of NAF active substances from OK sup.

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