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Effect of picibanil (OK432) on neutrophil-mediated antitumor activity: implication of monocyte-derived neutrophil-activating factors

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Summary. Picibanil (OK432), an extract from streptococci, has been widely utilized to treat malignant ascites and pleural effusions. The antitumor mechanism is believed to include complement-mediated neutrophil activation. Employing a flow-cytometric analysis of actin polymerization as an indicator of cell activation as well as a tumor proliferation assay, we have found that monocyte-derived neutrophil-activating factors were involved in OK432-induced neutrophil activation as well as antitumor activity. OK432-stimulated (0.1 KE/ml; 0.01 mg/ml) monocyte supernatants (OKMS) induced neutrophil actin polymerization and chemotaxis. OKMS were responsible for neutrophil-mediated inhibition of human leukemic (CEM) cell proliferation and stimulated neutrophils to produce superoxide in the presence of CEM leukemic cells at an effector/target ratio higher than 20/1. In contrast, OK432 alone, OK432-stimulated lymphocyte supernatants, or OK432-stimulated neutrophil supernatants had no effect on neutrophil activation or suppression of tumor cell proliferation. OK432 in combination with mononuclear cells also had no effect on the inhibition of CEM cell proliferation. Pretreatment of OKMS at 56°C for 30 min did not affect its ability to activate neutrophils, implying that complement activation is not responsible for the neutrophil activation. Supernatants from OK432-stimulated mononuclear cells, as determined by enzyme-linked immunosorbent assays and radioimmunoassays, contained high levels of interleukin-8 (IL-8; 1567 ± 145 pg/ml) and tumor necrosis factor (TNFα; 2105 ± 152 pg/ml), low levels of leukotriene B4 (800 ± 45 pg/ml) and IL-1β (180 ± 22 pg/ml), but interferon γ was not detectable. IL-1β, IL-8, and TNFα transcripts, undetectable in untreated monocytes, increased significantly after 30–60 min exposure to OK432. These results suggest that neutrophil-activating factors from monocytes or resident macrophages may play an important role in the OK432-induced neutrophil activation and antitumor activity.

Key words: OK432 – Neutrophil – Antitumor activity

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Introduction

Picibanil (OK432), an extract of *Streptococcus pyrogenes* A3, is a useful biological response modifier. It has been used effectively to treat malignant ascites and pleural effusions [8, 14–16, 28]. Neutrophils are involved in the OK432-mediated antitumor activity [8, 15, 16, 23, 28, 31]. Intraperitoneal and intrapleural administration of OK432 induces marked neutrophilic infiltration in 3–6 h [8, 16, 17]. Such neutrophilic recruitment has been suggested to involve OK432-induced complement activation [8, 10, 16, 17]. Inhibitors of arachidonic acid metabolism can block OK432-induced neutrophil activation [17], implying that eicosanoids may also play a role in the effects observed with OK432 treatments. Eicosanoids, however, may be produced by monocytes or macrophages in addition to neutrophils. Macrophage depletion by peritoneal lavage significantly suppresses endotoxin- or zymosan-mediated neutrophilic infiltration [24]. Therefore, it is unclear whether OK432 activates neutrophils directly or does so indirectly via paracrine factors produced by lymphocytes or monocytes. The current study demonstrates that OK432 induces neutrophil activation, chemotaxis, and antitumor activity via monocyte-derived neutrophil-activating factors.

Materials and methods

Preparations of leukocytes. Leukocytes were prepared from heparinized peripheral blood donated by healthy adult volunteers. Dextran (2%; T500, Pharmacia Fine Chemicals, Piscataway, N. J.) was mixed with the peripheral blood at a ratio of 1 : 4, and the syringes were incubated nozzle upward at 37°C for 30 min. Whole leukocytes containing neutrophils, monocytes and lymphocytes were harvested from the upper fraction, which represents leukocyte-enriched plasma. Polymorphonuclear cells (neutrophils) and mononuclear cells (MNC) were separated by a gradient centrifugation in Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, N. J.) at 1500 *g* for 15 min [32, 33]. Neutrophils were harvested from the cell pellet. Interface mononuclear leukocytes were collected, washed, and suspended at 5×10^6 cells/ml. In some experiments, monocytes and lymphocytes from the mononuclear leukocyte fraction were further separated by allowing the monocytes to adhere on a 24-well plastic plate at 37°C for 30 min. After this procedure, adherent cells were monocytes and non-adherent cells were lymphocytes [13]. Cells were suspended to 5×10^6 cells/ml in RPMI-1640 medium (Gibco) containing 5% fetal calf

serum for actin polymerization assay. In chemotaxis experiments, cells were suspended at 2×10^6 cells/ml in Hanks' balanced salt solution (HBSS; Gibco) containing 0.5% human serum albumin.

Measurement of actin polymerization. Actins in non-muscle cells exist mainly in the globulin form (G actin) in a resting state [3, 18]. During cell activation and movement, actins change rapidly between depolymerized forms (G actins) and polymerized (filamentous) forms (F actins) [4, 22]. Thus, changes in F actin content can reflect the status of cytoskeleton organization resulting from cell activation or locomotion [21]. Employing a flow-cytometric analysis of nitrobenzooxadiazole (NBD)/phallacidin (Molecular Probes, Eugene, Ore.) staining, in which phallacidin specifically binds to F actin [1, 2], the F actin content can be measured by the NBD fluorescence at 520-nm emission wavelength using 488-nm excitation light [12, 29]. Leukocytes (1.2 ml; 5×10^6 cells/ml) were placed into a microcentrifuge (1.5 ml polypropylene tube, Treff Laboratory Inc., Schweiz, Switzerland) and incubated with different concentrations (0, 0.001, 0.01, 0.05, 0.1, 0.2 KE/ml) of OK432 (1 KE = 0.1 mg streptococcal extract, Chugai Pharmaceutical Co., Tokyo, Japan) in a 37° C incubator with rotation at 8–10 cycles/min. After 0, 30, 60, 90, and 120 min, 0.2-ml samples of reaction mixtures were removed to be permeablized, fixed, and stained with an equal amount of NBD/phallacidin staining cocktail (7.4% formaldehyde, NBD/phallacidin (0.11 μM), and 100 μg/ml lysolecithin) at 37° C for 10 min. After washing twice in cold phosphate-buffered saline (PBS), cells were suspended in 0.5 ml PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, Calif.). In some experiments, neutrophils (5×10^6 cells/ml) were incubated with OK432-stimulated monocyte supernatants (OKMS), OK432-stimulated lymphocyte supernatant (OKLS) or neutrophil supernatants (OKNS) at 37° C for 30 s, 15 min and 30 min. OKMS, OKLS, and OKNS were prepared by incubating monocytes, neutrophils, and lymphocytes with OK432 (0.1 KE) at 37° C for 2 h. Forward scatter was simultaneously utilized to gate a cell population of specific size. Results were recorded as histograms of fluorescence versus cell number.

Chemotaxis assay. Chemotaxis was assessed in a 48-well microchemotaxis chamber (Neuropore Inc., Cabin John, Md.), modified from a previous description [11]. Lower chambers were filled with 30 μl HBSS, RPMI-1640 medium, OK432, OKMS, OKNS, or OKLS suspensions. Formylmethionyl-leucyl-phenylalanine (10 nM, Sigma Co.) was utilized as a positive control. Upper chambers were filled with 50 μl neutrophil suspensions (2×10^6 cells/ml). A 5-μm-pore-size filter (Millipore Co., Bedford, Mass.) was placed between the upper and lower chambers. Reactions were carried out in a humidified CO_2 incubator for 2 h. After incubation, filters were fixed in methanol and stained with hematoxylin. Neutrophils migrating completely through the filter were viewed at a 400× microscope field. Leukocytes in five random fields were counted. Each experiment included triplicate samples and reproduced at least three times.

Inhibition of tumor cell proliferation. Assessment of tumor cell proliferation was performed by a [H^3]thymidine (ICN Radiochemicals, Irvine, Calif.) incorporation assay as previously described [31]. The human leukemia cell line CEM (ATCC, Rockville, Md.) was cultured in RPMI-1640 medium containing 10% fetal calf serum. Reactions contained (a) 50 μl tumor suspension (1×10^5 cells/ml), (b) different concentrations of neutrophils or mononuclear cells to yield effector/target (E/T) ratios of 50/1, 20/1, 10/1, and 1/1, and (c) a series of stimuli including OK432, OKMS, OKLS, or OKNS. Control wells were incubated with tumor suspension alone, leukocyte suspension alone, or tumor suspension plus leukocyte suspensions without stimulus. Reactions were incubated in a CO_2 incubator at 37° C for 0.5 h before 1 μCi [H^3]thymidine was added to each well. After an additional 4 h of incubation, cells were collected and counted by a β counter. Each experiment was performed in triplicate and repeated three times. Data are presented as mean [H^3]thymidine incorporation (cpm).

Measurement of superoxide production. Detection of superoxide production was performed using a modification of the superoxide-dismutase-inhibitable cytochrome *c* reduction assay [32]. A 50 μl sample of neutrophil suspension (2×10^6 cells/ml) and 20 μl cytochrome *c* (80 mM; Sigma) were added to each well of a 96-well microplate containing a total volume of 200 μl. Different concentrations of tumor cells were added to yield effector/target ratios of 20/1 and 10/1. Reactions were incubated in a water bath shaker at 37° C for 30 min. All the reactions were run in triplicate and in two sets of experiments each, with and without superoxide dismutase (50 μg/ml). Results were measured with an enzyme-linked immunosorbent assay (ELISA) reader at 550 nm. Data were calculated using the molar absorption coefficient, $2.11 \times 10^4\ M^{-1}cm^{-1}$, for cytochrome *c* reduction [32].

Detection of neutrophil activating factors. Interleukin-1 (IL-1β), IL-8, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) were measured by ELISA assays. ELISA kits for IL-1β, TNFα, and IFNγ were purchased from Genzyme Co. (Boston, Mass.), while IL-8 ELISA kits were purchased from Quantikine (R & D Systems Inc., Minneapolis, Minn.). Leukotriene B4 (LTB4), a known neutrophil-activating factor among eicosanoids, was assayed by radioimmunoassay (RIA) [16]. Values were based on interpolation from a standard curve generated by samples of known concentration provided with each ELISA and RIA kit. Results were expressed as means ± standard deviation for three experiments.

Detection of cytokine mRNA expression. MNC (2×10^6/ml) were incubated with or without OK432 (0.1 KE) in a 37° C incubator and rotated at 8–10 cycles/min for 15, 30, 60, 90, and 120 min. Total cellular RNA was purified by a guanidine isothiocyanate/cesium chloride procedure [5]. RNA was subjected to electrophoresis in 1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized to one of the following ^{32}P-labeled cDNA probes: (a) the 0.6-kb (0.6×10^3 bases) *Eco*RI insert of human IL-8 cDNA purified from pUC19 plasmid [20], kindly provided by Dr. Joost J. Oppenheim (Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick, Md.), (b) the 0.6-kb *Bam*HI/*Sma*I insert of human IL-1β cDNA purified from YEpsec1 plasmid (ATCC), (c) the 1.1-kb *Pst*I insert of human TNFα cNDA purified from pE4 plasmid [30], and (d) the 2.0-kb *Pst*I insert of a chicken β-actin gene [7]. Hybridizations were carried out at 42° C for 16 h in 50% (v/v) formamide, 2× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate), 1× Denhardt's solution, 0.1% SDS (sodium dodecyl sulfate), and 200 μg/ml salmon sperm DNA [25]. Filters were washed twice in 2× SSC/0.1% SDS at room temperature and twice in 0.1× SSC/0.1% SDS at 56° C for 30 min and exposed to Kodak X-OMAT X-ray film using an intensifying screen.

Statistics. Unpaired Student's *t*-test was employed to test the significance of comparisons of effects in the presence or absence of a stimulus.

Results

Effect of OK432 on leukocyte activation

Analysis of actin polymerization has been used to monitor leukocyte activation [21]. Actin polymerization is associated with cellular conformational change during activation. Employing whole leukocyte suspensions, we found that resting monocytes displayed higher F actin levels than neutrophils and lymphocytes, whereas neutrophils, but not monocytes or lymphocytes, displayed an increase in F actin levels after stimulation with OK432 (0.1 KE). As shown in Fig. 1 A, the enhancement of actin polymerization in neutrophils by OK432 was time-dependent, becoming maximal at 90 min. On the basis of 120-min exposure, the OK432-mediated enhancement of actin polymerization was concentration-dependent and maximal at a level between 0.05 KE and 0.10 KE (Fig. 1 B). These results suggest that OK432 activates neutrophils within 2 h.

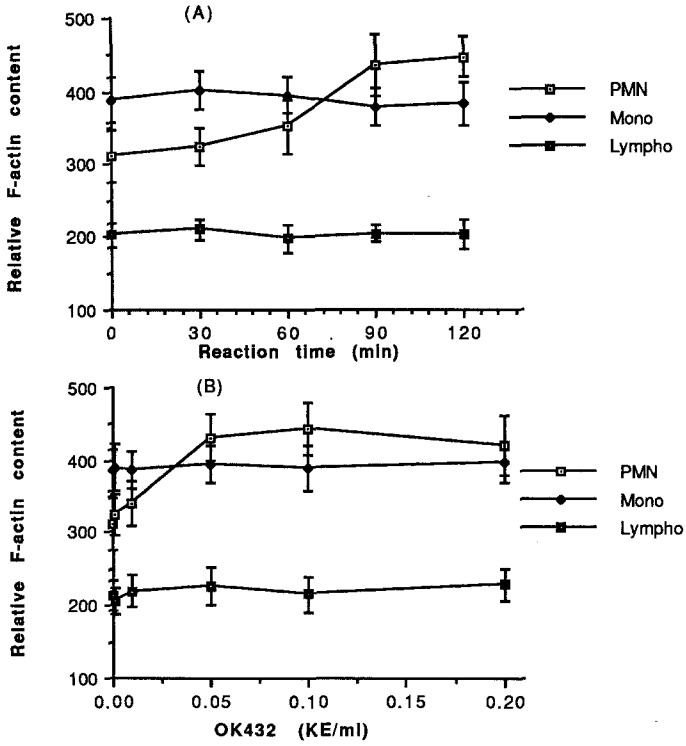

Fig. 1 A, B. Effects of OK432 on the activation of different leukocytes as determined by actin polymerization. **A** Whole peripheral leukocytes (5×10^6 cells/ml) including neutrophils, monocytes and lymphocytes were incubated with OK432 (0.1 KE) at 37° C for the indicated time. **B** OK432 induced a dose-dependent effect on the activation of neutrophils present in the whole peripheral leukocytes. The response reached a plateau when the concentration of OK432 approached 0.05 KE. *PMN*, polymorphonuclear cells (neutrophils)

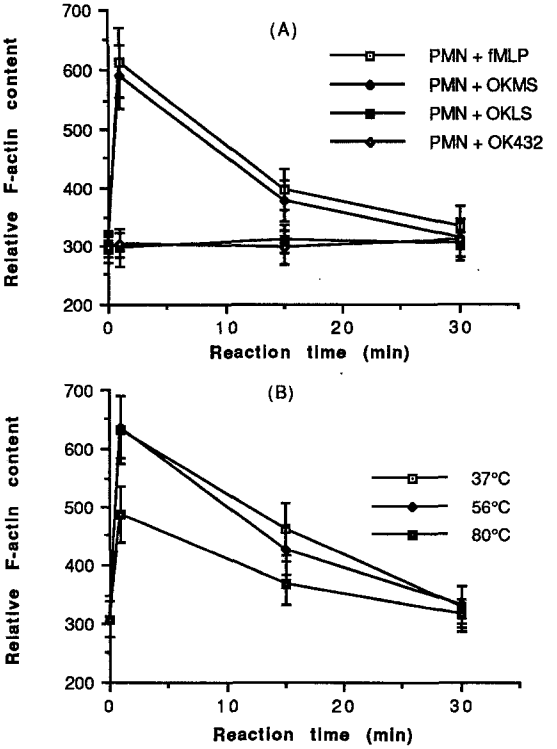

Fig. 2 A, B. Effect of OK432-stimulated monocyte supernatant (*OKMS*) on the neutrophil activation. **A** OK432 (0.1 KE) alone did not induce neutrophil activation as determined by actin polymerization. OK432-stimulated lymphocyte supernatant (*OKLS*; 10%, v/v) also had no effect on the neutrophil activation. OKMS (10%, v/v) induced a rapid actin polymerization as potent as that induced by fMet-Leu-Phe (*fMLP*; 0.1 μM). **B** Action of OKMS on the actin polymerization of neutrophils was heat-stable. OKMS pretreated at 80° C for 30 min, but not that treated at 56° C for 30 min, showed reduced ability to induce actin polymerization

Experiments were next performed to determine if OK432 activated neutrophils directly or indirectly. Whole-blood leukocytes were separated into polymorphonuclear (neutrophils) and mononuclear leukocytes (MNC) by a gradient centrifugation in Ficoll-paque. Monocytes and lymphocytes in the MNC were further purified by allowing monocytes to adhere at 37° C for 30 min. As illustrated in Fig. 2A, OK432 (0.1 KE) alone did not induce actin polymerization in purified neutrophils. OKMS at a concentration of 10% (v/v), but not OKLS or OKNS, significantly promoted actin polymerization of purified neutrophils within 30 s (Fig. 2A). OKMS preincubated at 56° C for 30 min did not reduce its action on actin polymerization. Pretreatment of OKMS at 80° C for 30 min reduced its activity by 50% (Fig. 2B). These results suggest that OK432 activates neutrophils indirectly via heat-stable paracrine factors derived from monocytes.

Effect of OK432 on neutrophil chemotaxis

OK432 alone had no effect on the induction of neutrophil chemotaxis. OKMS, but not OKNS or OKLS, induced neutrophil chemotaxis. The stimulation of neutrophil chemotaxis by OKMS was dose-dependent (Fig. 3).

Effects of OK432 on the CEM tumor cell proliferation

Neutrophils alone or in combination with OK432 (0.1 KE) did not inhibit proliferation of CEM leukemic cells. As shown in Fig. 4A, when an E/T ratio was set at 50/1, 10% (v/v) OKLS and OKNS had no effect on the suppression of CEM cell proliferation. In contrast, OKMS showed a significant effect on the neutrophil-mediated suppression of tumor cell proliferation in a dose-dependent fashion. In the presence of 10% (v/v) OKMS, neutrophils suppressed the proliferation of CEM leukemic cells at neutrophil/tumor cell ratios of as low as 20/1 (Fig. 4B).

Effects of OK432 on the production of superoxide by neutrophils

CEM leukemic cells alone did not produce superoxide. Untreated neutrophils (5×10^5 cells/well) released 2.05 ± 1.20 nmol superoxide/10^6 cells in 30 min. OK432 (0.1 KE) or OKMS (10%, v/v) alone did not augment superoxide production by neutrophils. However, when CEM leukemia cells were added to the reactions at the E/T ratio of 20/1, OKMS significantly enhanced superoxide production by neutrophils over fourfold as shown in Fig. 5. At a lower E/T ratio (10/1) OKMS-stimulated neutrophil

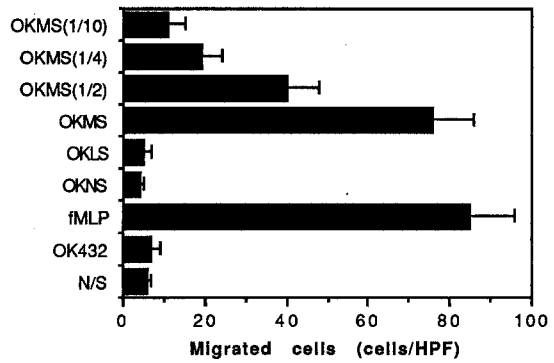

Fig. 3. Effect of OKMS on the neutrophil chemotaxis. OK432 alone did not show any chemotactic activity for neutrophils. OKLS and OKNS had no effect on the neutrophil chemotaxis. OKMS induced neutrophil chemotaxis in a dose-dependent fashion. A well-known chemoattractant, fMet-Leu-Phe (*fMLP*; 10 nM), was run as a control. *HPF*, high-power field

enhancement of superoxide production was less (5.52 ± 0.98 nmol/10^6 cells in 30 min), but still greater than the control.

Effects of OK432 on production of monocyte-derived neutrophil-activating factors

Neutrophil-activating factors, including LTB4, IL-1β, IL-8, TNFα, and IFNγ, in the OKMS were measured by RIA and ELISA assays. As shown in Table 1, Stimulation of MNC by OK432 for 2 h resulted in the secretion of IL-8 (1567 ± 145 pg/ml), TNFα (2105 ± 152 pg/ml), LTB4 (800 ± 45 pg/ml) and IL-1β (180 ± 22 pg/ml), but not IFNγ production. Production of these neutrophil-activating factors by OK432 stimulation of MNC was not affected by the addition of polymyxin B (10 μg/ml), suggesting that endotoxin contamination did not account for the observed effects.

Experiments were next performed to study the kinetics of IL-1β, IL-8, and TNFα production from MNC after exposure to OK432. IL-8 and TNFα were detectable as early as 30 min after OK432 stimulation, while IL-1β levels were still low after OK432 stimulation for 2 h (Fig. 6). Northern blot analyses of MNC mRNA using

Table 1. Production of neutrophil-activating factors (NAF) by mononuclear cells (MNC) stimulated by OK432 at 37° C for 2 h[a]

NAF	MNC + OK432	MNC + N/S	MNC + PMB + OK432
LTB4 (pg/ml)	800± 45	70±16	Not done
IL-1β (U/ml)	180± 22	12± 8	Not done
TNFα (pg/ml)	2105±152	120±46	2075±185
IFNγ (U/ml)	Undetectable	Undetectable	Not done
IL-8 (pg/ml)	1567±145	54±30	1428±203

[a] OK432: 0.1 KE; N/S, normal saline; PMB, polymyxin B (10 μg/ml). Measurement of leukotriene B4 (LTB4) was performed by a radioimmunoassay, while interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) interferon γ (IFNγ) and IL-8 were measured by enzyme-linked immunoassays

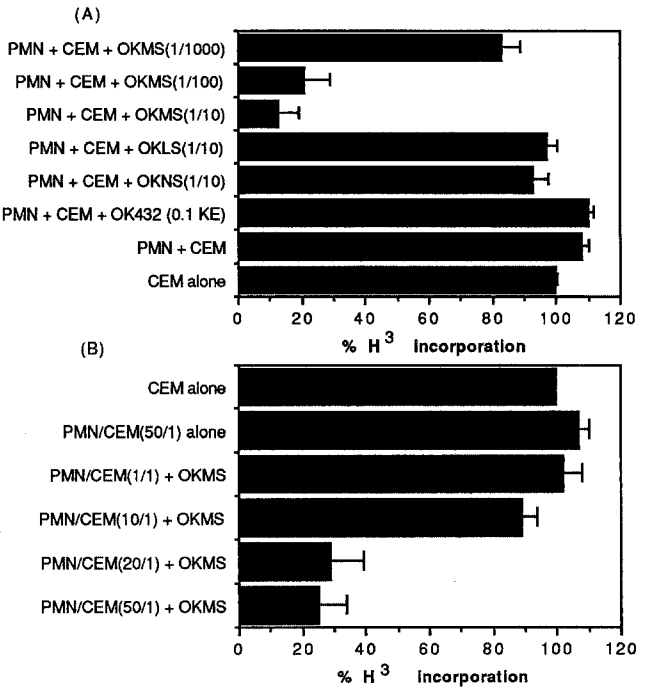

Fig. 4. Effects of OK432 and OKMS on the inhibition of tumor (CEM) cell proliferation, as determined by a [H^3]thymidine incorporation assay. **A** When the neutrophil/CEM cell effector/target (E/T) ratio was set at 50/1, OKLS and OKNS at 1/10 (v/v) concentration or OK432 (0.1 KE/ml) alone did not promote neutrophil-mediated antitumor activity. Addition of OKMS at 1/10 and 1/100 significantly suppressed the proliferation of CEM tumor cells. **B** Different E/T ratios had different effects on the OKMS-induced antitumor activity. OKMS-induced antitumor activity did not appear unless the E/T ratio was set higher than 20/1

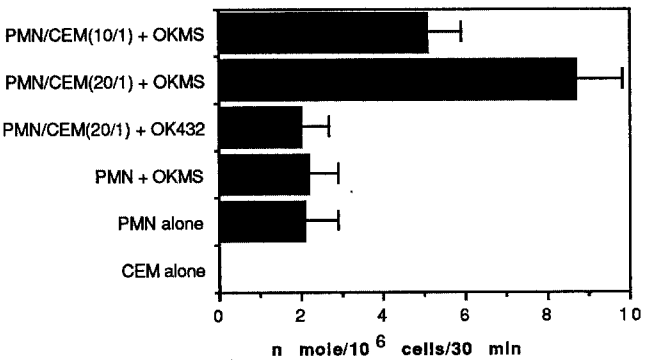

Fig. 5. Effect of OKMS on the superoxide production by neutrophils co-stimulated with CEM tumor cells. OK432 or OKMS alone had no effect on the superoxide production by neutrophils. OKMS did have a significant effect on superoxide production against CEM tumor cells by neutrophils ($P < 0.05$)

IL-1β, TNFα, and IL-8 probes are shown in Fig. 7. IL-1β and IL-8 transcripts (each approximately 1.8–2.0 kb), which were undetectable in untreated cells, appeared after 30 min of OK432 treatment and were maximal by 90–120 min. TNFα transcripts (2.3 kb) were also undetectable in untreated cells, appeared somewhat later (60 min), and were also maximal at 90–120 min. OK432 treatment had no effect on actin transcript levels, suggesting that the effect on cytokine mRNA levels was specific and not due to differences in the amount of RNA loaded or

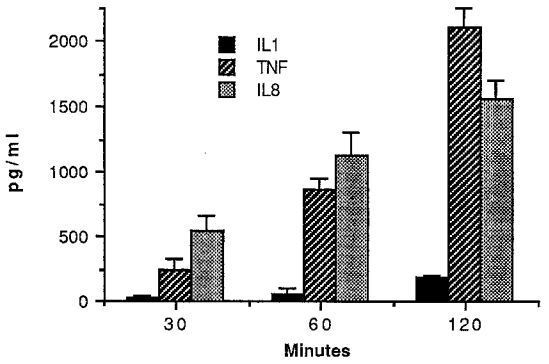

Fig. 6. Production of interleukin-1β (*IL1*), interleukin-8 (*IL8*), and tumor necrosis factor α (*TNF*) by mononuclear cells (MNC) stimulated by OK432 for different times. MNC (2×10^6 cells/ml) were incubated with OK432 (0.1 KE/ml) at 37° C for 30, 60, and 120 min. Results were determined by enzyme-linked immunosorbent assays. Data shown are calculated from two duplicate experiments and presented as means ± standard errors

transferred in each lane. Moreover, MNC treated in medium alone for 2 h did not display IL-1β, IL-8, and TNFα transcripts. Since both the IL-8 transcript and protein appeared at or before the time of induction of IL-1β and TNFα gene products (Figs. 6 and 7), it is unlikely that IL-8 production is secondary to that of TNFα or IL-1β production.

Discussion

Picibanil (OK432) has been utilized to treat malignant ascites and pleural effusions [8, 14–16, 28]. Clinical studies demonstrated that injection of OK432 into the peritoneal space rapidly recruited a large number of neutrophils in 3–6 h [8, 16, 17]. Animal studies suggested that complement-mediated neutrophil activation was apparently involved in the OK432-induced antitumor activity [10, 16, 17]. In this study, we have found that OK432 alone did not directly activate neutrophils in 2 h; however, monocyte-derived neutrophil-activating factors induced by OK432 were involved in the neutrophil activation and antitumor activity. These results clearly indicate that monocytes and macrophages may play an important role in the OK432-induced neutrophil-mediated antitumor activity.

We found that neutrophils were efficient effector cells against the human CEM leukemic cells in the presence of the monocyte-derived neutrophil-activating factors induced by OK432. Neutrophils, residing in close proximity to sites of leukemic cell proliferation in the bone marrow and blood, may be ideal effector cells to resist leukemia in the presence of monocytes and OK432. Taken together, these results suggest that OK432 may have a potential for treating patients with leukemia. This notion is supported by a recent report describing successful treatment of a patient with T cell leukemia/lymphoma by OK432 [19].

Neutrophil-mediated antitumor activity is a well-recognized phenomenon. The mechanisms by which neutrophils participate in the antitumor activity remain to be determined. Antibody-dependent cell cytotoxicity (ADCC) is one such proposed mechanism [6, 9]. On the basis of

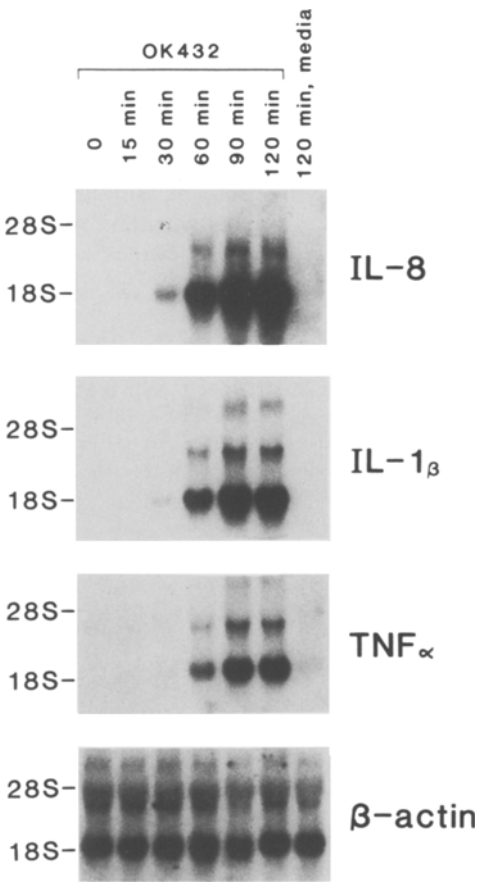

Fig. 7. Effects of OK432 on the induction of cytokine mRNA levels in MNCs. Northern blot analysis of RNA levels was performed in MNC after treatment by OK432 (0.1 KE). Total cellular RNA (20 μg/ml) was hybridized to ^{32}P-labeled IL-1β, IL-8, TNFα, and β actin cDNA probes

current studies, it appears unlikely that the neutrophil-mediated antitumor activity induced by OK432 was ADCC since no tumor-specific antibody was present in the reaction mixtures. On observing that tumor cells from OK432-treated ascites form rosettes around with seven to ten neutrophils, Saji et al. [23] have suggested that administration of OK432 may increase the cohesion of tumor cells with neutrophils. Results from our study were compatible with this hypothesis. OK432 induced monocytes to secrete neutrophil-activating factors such as IL-1β, IL-8, TNFα, and LTB4, which can promote the expression of adhesion molecules on neutrophils. Neutrophil-activating factors in the OKMS significantly enhanced superoxide production by neutrophils co-cultured with tumor cells. Taken together, these data suggest that monocyte-derived neutrophil-activating factors in the OKMS may be able to recruit neutrophils, enhance the adhesion of neutrophils with tumor cells, and cause superoxide production resulting in tumor cell damage. Furthermore, OK432-induced neutrophil activation may release a chemoattractant for natural killer cells, which may also be involved in the antitumor effect of OK432 [10].

Monocytes/macrophages produce IL-8 in response to endotoxin (lipopolysaccharide), TNFα, or IL-1β [26, 27]. In this study, we found that OK432-induced IL-8 produc-

tion did not appear to be secondary to either lipopolysaccharide, IL-1β or TNFα production since polymyxin B, an inhibitor of lipopolysaccharide mediated cytokine production, did not block OK432-induced IL-8 production and OK432-induced IL-8 mRNA expression present at or before IL-1β, and TNFα mRNA expression. In summary, OK432 stimulates neutrophil activation and antitumor activity via a monocyte-dependent mechanism. This mechanism may involve the elaboration of cytokines such as IL-8, TNFα, IL-1β, and LTB4.

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