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The Mechanisms of Cytotoxicity to Tumor Cells by Polymorphonuclear Leukocytes Stimulated with Cytokines

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The mechanisms of tumor cytotoxicity of rat polymorphonuclear leukocytes (PMN) activated with cytokine(s) were studied with the use of supernatants from a rat myelomonocytic leukemia cell line, WRT-7, incubated in the presence of bacterial lipopolysaccharide (LPS) (LPS WRT-7 sup) as a source of cytokine. Rat peritoneal PMN treated with LPS WRT-7 sup showed cytostasis from 3 hr after the start of incubation, while significant cytolysis was first observed after 24 hr. When target tumor cells were separated from PMN at 6 or 12 hr after the start of the assay, ^{3}H-UdR release from the separated target cells comparable to that from the group incubated with PMN for the whole assay time of 40 hr was observed during the following incubation, which indicates that priming for subsequent lysis occurs at a relatively early stage of the assay. None of various scavengers of active oxygens, inhibitors of heme enzymes, and inhibitors of neutral proteinases inhibited cytolysis mediated by PMN stimulated with LPS WRT-7 sup. Heparin inhibited PMN cytolysis only when it was added within 1 hr after the start of the assay. Fractionation of heparin by ion exchange chromatography showed a parallelism between the negative charge and the inhibitory effect of heparin on PMN cytotoxicity.

Key words: Polymorphonuclear leukocyte — Tumor cytotoxicity — Cytokine

Recently, polymorphonuclear leukocytes (PMN)[*4] have been considered by several workers as possible effector cells in tumor resistance, especially when tumor-bearing hosts are treated with several biological response modifiers (BRM). Lichtenstein *et al.* have demonstrated that mouse embryonal teratocarcinoma can be successfully treated by administration of *Corynebacterium parvum*,[1–3] and in this system, they showed that PMN are important effector cells. Morikawa *et al.* have demonstrated in the mouse system that linear β-1,3-D-glucan obtained from *Alcaligenes faecalis* strongly activates tumoricidal PMN.[4–6] We also have demonstrated that intraperitoneal injection of a streptococcal preparation, OK-432, induces PMN cytotoxicity to tumor cells.[7] On the other hand, we have reported that culture supernatants of rat spleen cells stimulated with concanavalin A or culture supernatants of a T cell leukemia cell line, Jurkat, render rat PMN cytotoxic.[8–10]

The mechanisms of oxygen-dependent PMN cytotoxicity have been well documented, that is, superoxide,[11] the myeloperoxide-H_2O_2-halide system,[12–14] or H_2O_2 alone[15–17] has been shown to be the effector, depending on the systems used. In the case of cytotoxicity by PMN stimulated with BRM, H_2O_2[3, 6] and superoxide[3] have also been identified as effector molecules. The characteristic of the oxygen-dependent cytotoxicity is that cytolysis of target cells occurs in a short time of incubation (from two to eight hours) depending on the systems used. On the other hand, the kinetics of cytotoxicity mediated by PMN stimulated with cytokines

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[*4] Abbreviations: PMN, polymorphonuclear leukocytes; WRT-7, rat myelomonocytic leukemia cell line; LPS., bacterial lipopolysaccharide; LPS WRT-7 sup, culture supernatant of WRT-7 stimulated with LPS; c-IMDM, Iscove's modified Dulbecco's medium supplemented with 5 μg/ml of transferrin, 5 μg/ml of insulin, 100 μg/ml of soybean lipid, 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 3 mg/ml of $NaHCO_3$; MEM, Eagle's minimal essential medium; NAF, neutrophil activating factor; E/T, effector to target; SOD, superoxide dismutase.

are different from those of PMN cytotoxicity whose effector molecules are the active oxygens mentioned above. As already reported elsewhere, cytostasis by PMN pretreated with cytokines appeared at an early phase of the assay (3 to 6 hr), and reached a plateau at 12 hr of incubation, while cytolysis firstly appeared after incubation for more than 24 hr.[8, 18] The difference in the kinetics of PMN-mediated tumor cytolysis led us to speculate that the mechanisms of cytotoxicity by PMN stimulated with cytokines might be different from those of PMN cytotoxicity, whose effector molecules are active oxygens. To clarify this point, in the present study we mainly used a rat myelomonocytic leukemia cell line, WRT-7, which produced factor(s) that rendered PMN cytotoxic following the differentiation to monocytes/macrophages by bacterial lipopolysaccharide (LPS).

In the present paper, we describe the relationship between cytostasis and cytolysis mediated by cytokine-treated PMN, and the active oxygen non dependency of this PMN cytolysis. We will tentatively call the factor(s) that renders PMN cytotoxic the neutrophil activating factor (NAF). We will use the term "cytotoxicity" in a broad sense that includes "cytostasis" and "cytolysis."

Materials and Methods

Animals and Tumor Cells Donryu SPF rats (male, 6–8 weeks) were purchased from Shizuoka Laboratory Co., Ltd. (Shizuoka), and kept in a bacteria-free condition using "clean racks" until use. We used them as a source of effector neutrophils. Tumor cell lines used as target cells in the cytotoxicity assay were RL♂-1 (an X-irradiation-induced lymphoma of BALB/c mice), P 815 (a mastocytoma of DBA/2 mice), YAC-1 (a Molony leukemia virus-induced lymphoma of A/Su mice), KMT-17 (a methylcholanthrene-induced fibrosarcoma of WKA rats), MM 46 (a breast cancer of C3H mice), and K 562 (a human erythroleukemia cell line). They were maintained in *in vitro* culture in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 100 U/ml of penicillin-G, 100 μg/ml of streptomycin, 2 mg/ml of $NaHCO_3$, 3 mg/ml of HEPES (Dojin Laboratory Co., Ltd., Kumamoto), and 10% heat-inactivated fetal calf serum (FCS) (Gibco Lab., Chagrin Falls, OH), and passaged every three days. Routinely, we used RL♂-1 as a target tumor cell. The WRT-7 cells (a myelomonocytic leukemia cell line of WKA rats induced by Rauscher leukemia virus) were maintained in *in vitro* culture in Iscove's modified Dulbecco's medium (Gibco Lab.) supplemented with 5 μg/ml of transferrin (Sigma, St. Louis, MO), 5 μg/ml of insulin, 100 μg/ml of soybean lipid (Sigma), 100 U/ml of penicillin G, 100 μg/ml streptomycin, and 3 mg/ml of $NaHCO_3$ (complete IMDM: c-IMDM), and passaged every three days.

Preparation of Culture Supernatant of WRT-7 Cells Stimulated with LPS Various numbers of WRT-7 cells in c-IMDM were cultured with various doses of LPS (Type V, Sigma) at 37° in a 5% CO_2-in-air atmosphere for 40 hr. Thereafter, culture supernatants were collected by centrifugation at 3000 rpm for 30 min at 4°, filtered with a Millex-HA (0.45 μm; Millipore Co., Bedford, MA), and stored at −70° (LPS WRT-7 sup) until used for cytotoxicity assay. As another NAF source, we used culture supernatant from rat spleen cells stimulated with a streptococcal preparation, OK-432 (OK sup). The details concerning OK sup were presented in a previous paper.[18]

Preparation of PMN Suspensions PMN were obtained from rat peritoneal exudate cells (PEC) as described in detail elsewhere.[8–10, 18] Briefly, 10 ml of 3% proteose-peptone (Difco Lab., Detroit, MI) was injected intraperitoneally twice about 15 and 3 hr before the cell harvest. Collected PEC were placed on Ficoll-Isopaque solution (specific gravity, 1.090; Pharmacia Fine Chemicals AB, Uppsala, Sweden), and centrifuged at 2200 rpm for 30 min at 4°. After centrifugation, the cell pellets at the bottom which contained more than 99% neutrophils determined by May-Giemsa staining were gently collected by means of Pasteur pipets.

***In vitro* Activation of Rat Neutrophils by NAF and Cytotoxicity Assay** Various numbers of PMN in 100 μl were incubated with the same amount of LPS WRT-7 sup (in some experiments OK sup) for 3 hr at 37° in a Falcon Micro Test Plate II 3042 (Falcon Plastics, Los Angeles, CA). Unless otherwise stated, 5 μg/ml polymyxin B was added to each reaction mixture to block PMN activation induced by contaminated LPS.[10] After incubation, treated PMN were washed three times with MEM (Nissui) and used for the following cytotoxicity assay.

Target cells in cytotoxicity assay were RL♂-1, unless otherwise stated. a) Cytolysis assay. The details are described elsewhere.[8, 18] Briefly, 2×10^4 target cells prelabeled with ^{3}H-UdR were mixed with activated PMN, and the reaction mixtures were incubated at 37° in a 5% CO_2-in-air atmosphere for various periods of time (routinely, 40 hr). At the end of the culture, the radioactivity of the supernatant of each well was counted with a liquid scintillation counter (Aloka Liquid Scintillation System, LSC 751). All assays were done in

triplicate. Percent specific lysis was calculated by applying the following formula:

$$\% \text{ specific lysis} = \left(\frac{\text{cpm of experimental group} - \text{cpm of spontaneous release}}{\text{cpm of maximum release} - \text{cpm of spontaneous release}} \right) \times 100.$$

In some experiments, PMN were removed from the reaction mixtures during cytolysis assay by the Percoll (Pharmacia Fine Chemicals) discontinuous gradient method, and subsequent ^{3}H-UdR release from tumor cells was observed. The Percoll gradient consisted of 70%, 65%, 60%, 50%, and 30% concentrations. Reaction mixtures were placed on a Percoll gradient and centrifuged at 1500 rpm for 1 hr at room temperature. Target cells were situated at the third interface. b) Cytostasis assay. The details are described elsewhere.[8-10, 18] Briefly, *in vitro* activated PMN were mixed with 1×10^4 target cells, and the reaction mixtures were incubated at 37° in a 5% CO_2-in-air atmosphere for various times. During the final 6 hr of incubation, the cells were pulse-labeled with 1 μCi/ml of ^{3}H-TdR. All assays were done in triplicate. The percent inhibition was calculated by applying the following formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{cpm of experimental group}}{\text{cpm of nontreated PMN}} \right) \times 100.$$

Fractionation of Heparin Heparin sodium solution (1 mg = 140 units) was fractionated on DEAE-Sephadex A-25 (Pharmacia) with the use of an 8× 40 mm column. The DEAE-Sephadex A-25 column was equilibrated with 0.5*M* NaCl, 0.02*M* imidazole buffer, pH 7.35. Thirty-five milligrams of heparin was applied to this column and the elution was performed with a stepwise gradient from 0.5*M* to 1.5*M* NaCl by using 20 ml each of 0.2, 0.4, 0.6, 0.8 and 1.0*M* NaCl in the imidazole buffer. Each fraction was 2 ml. Three major peaks were observed, and they were collected as Fr. I, Fr. II and Fr. III. These three fractions were dialyzed against RPMI-1640 medium at 4° for about 24 hr and used for the assay. Concentrations of heparin were determined by means of the standard carbazole reaction for uronic acid equivalents.

Reagents Catalase, SOD, sodium azide, and potassium cyanide were purchased from Sigma Chemicals. L-Histidine and D-mannitol were from Wako Pure Chemical Industries (Osaka). Heparin sodium was from Novo Industries (Copenhagen, Denmark). Aprotinin was from Bayer Co., Ltd. (Leverkusen, Germany).

Statistical Methods Statistical analysis was performed with Student's *t*-test.

Results

Tumor Cytolysis by PMN Treated with Supernatants of WRT-7 Cells Cultured in the Presence of LPS WRT-7 cells were morphologically changed to macrophages when they were incubated for 40 hr in the presence of LPS at doses of more than 0.2 μg/ml, as already reported by Fujii *et al.*[19] (data not shown). We examined the NAF activity of supernatants of WRT-7 cultured in the presence of LPS (LPS WRT-7 sup). LPS WRT-7 sup showed NAF activity, while supernatants from WRT-7 cultured in the absence of LPS did not augment PMN cytolysis. To evaluate

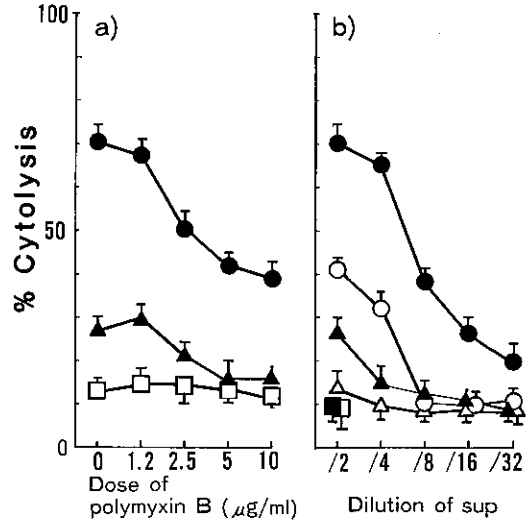

Fig. 1. NAF activity of LPS WRT-7 sup. LPS-WRT 7 sup was obtained after 40 hr of culture at 37° in the presence of 1 μg/ml LPS. WRT-7 sup was obtained under the same culture conditions in the absence of LPS. PMN were treated with LPS WRT-7 sup, WRT-7 sup, or LPS itself for 3 hr at 37° in the presence or absence of polymyxin B. Final dilution of each sup=1/2, assay time=40 hr, E/T ratio=50:1, bars=SEM. a) Various doses of polymyxin B were added at the time of PMN activation by LPS WRT-7 sup (●), WRT-7 sup (□), or LPS in c-IMDM (▲). b) PMN were treated with serially diluted supernatant. LPS WRT-7 sup (○), WRT-7 sup (□), LPS in c-IMDM (△) in the presence of polymyxin B (5 μg/ml). LPS WRT-7 sup (●), WRT-7 sup (■), LPS in c-IMDM (▲) in the absence of polymyxin B. $P<0.01$ between the LPS WRT-7 sup group and LPS in the c-IMDM group at all points examined in Fig. 1a.

the effect of LPS in LPS WRT-7 sup, we examined the NAF activity of LPS itself. Treatment of PMN with LPS itself augmented PMN cytolysis to some extent, but addition of polymyxin B at doses of more than 5 μg/ml abolished the PMN activation by LPS (Fig. 1). NAF activity of LPS WRT-7 sup was also diminished depending on the dose of polymyxin B added. However, LPS WRT-7 sup showed NAF activity even in the presence of polymyxin B at doses which completely inhibited the NAF activity of LPS. This result indicates that LPS WRT-7 sup contained NAF other than LPS (Fig. 1a). Tumor cytolysis by PMN treated with the agents mentioned above was dependent on the concentration of the agents used (Fig. 1b). To obtain the optimal conditions for production of NAF by WRT-7 stimulated with LPS, various numbers of WRT-7 cells were incubated in the presence of LPS. The optimal cell density required for sufficient NAF production was more than 1×10^5/ml (data not shown). When WRT-7 cells were stimulated with 5 μg/ml LPS, PMN cytolysis induced by stimulation with LPS itself was not completely inhibited to the level of the control group, even in the presence of polymyxin B in the assay. Thus, in the following experiments 2×10^5/ml WRT-7 cells were incubated for 40 hr in the presence of 1 μg/ml LPS, and the activation of PMN by culture supernatants was performed in the presence of 5 μg/ml polymyxin B. Cytolysis by PMN treated with LPS WRT-7 sup was dependent on the concentration of the sup and the number of PMN used (data not shown). NAF activity of LPS WRT-7 sup was canceled only by 100°, 5 min treatment, and was stable to treatments such as 60°, 30 min, pH 2 or pH 10 and freezing & thawing. This result indicates that NAF in LPS WRT-7 sup was different from NAF partially purified from concanavalin A-stimulated rat spleen cells.[9] Further, this cytotoxic effect of PMN possessed target selectivity, that is, P 815 and YAC-1 as well as RL♀-1 were highly susceptible, and KMT-17, MM 46 and K 562 were weakly susceptible (Table I).

Kinetics of Cytolysis and Cytostasis by PMN Treated with LPS WRT-7 Sup We next examined the kinetics of cytolysis and cytostasis mediated by LPS WRT-7 sup-treated PMN. Cytolysis was first observed at 24 hr after the start of the assay and reached a plateau at 36 hr (Fig. 2a). On the other hand, cytostasis was observed at a relatively early stage of the assay, and incubation of 6 to 12 hr was sufficient for peak cytostasis, whereas at this time significant cytolysis was not detected (Fig. 2b). In this experiment, cytolysis and cytostasis of PMN induced by LPS itself were not completely abrogated even in the presence of polymyxin B at PMN activation. Similar results were obtained in some other experiments. To clarify the mechanisms of the discrepancy in the kinetics of cytolysis and cytostasis mediated by PMN pretreated with LPS WRT-7 sup, the following experiments were performed.

PMN pretreated with LPS WRT-7 sup were mixed with ^{3}H-UdR-labeled target

Table I. Target Selectivity in Cytolysis of PMN Treated with LPS WRT-7 Sup[a]

Target cells	% Net lysis[b]					
E/T ratio:	50:1			25:1		
Dilution of sup:	1/2	1/4	1/8	1/2	1/4	1/8
RL♀-1	42.7	52.1	34.3	55.1	39.1	33.4
P 815	35.0	16.1	5.5	26.5	14.3	8.9
MM 46	5.8	1.5	0.7	3.6	2.5	−0.6
YAC-1	33.2	18.0	6.6	27.5	24.3	11.2
K 562	6.3	0.7	−0.2	1.5	1.2	1.2
KMT-17	9.6	1.3	1.1	6.3	0.6	−0.6

a) PMN were pretreated for 3 hr with LPS WRT-7 sup or LPS alone in the presence of 5 μg/ml polymyxin B. Assay time: 40 hr.
b) % net lysis = (% cytolysis of LPS WRT-7 sup-treated PMN) − (% cytolysis of LPS in c-IMDM-treated PMN).

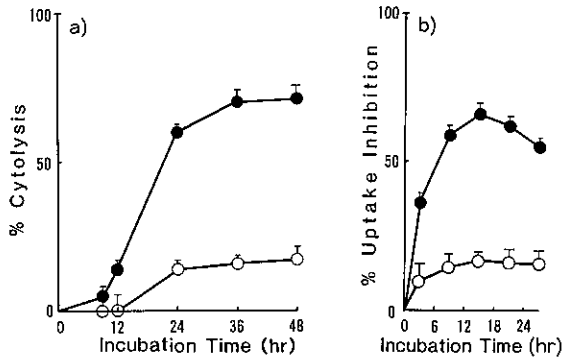

Fig. 2. Kinetics of cytotoxicity mediated by LPS WRT-7 sup-treated PMN. Final dilution of each sup = 1/4, E/T ratio = 50:1, bars = SEM a) Cytolysis assay. b) Cytostasis assay. LPS WRT-7 sup (●), LPS in c-IMDM (○).

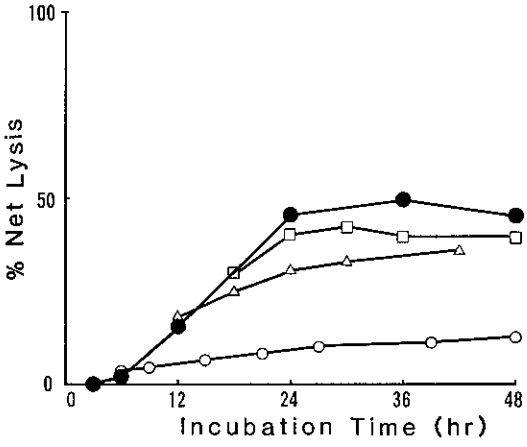

Fig. 3. ^{3}H-UdR release from target tumor cells after separation from LPS WRT-7 sup-treated PMN. Target tumor cells were separated by using Percoll from pretreated PMN 3 hr (○), 6 hr (△), and 12 hr (□) after the start of the co-culture. After separation, target cells were incubated in the absence of PMN for various periods of time. (●): Percent net lysis mediated by LPS WRT-7 sup-treated PMN without separation. Percent net lysis = (% cytolysis of LPS WRT-7 sup-treated PMN) − (% cytolysis of LPS in c-IMDM-treated PMN). E/T ratio = 50:1. Percent cytolysis of LPS in c-IMDM: 0.4% (3 hr), 8.6% (6 hr), 12.5% (12 hr), 16.1% (24 hr), 20.2% (36 hr), 31.2% (48 hr). $P < 0.01$ between LPS WRT-7 sup group and LPS in c-IMDM group at each point after 12 hr assay time.

tumor cells, and the mixtures were incubated at 37°. At various points of the incubation, the target tumor cells were separated from PMN by the Percoll gradient method. The recovery of the tumor cells was approximately 70%, and the viability of the separated tumor cells was not significantly different from that of the original tumor cells from the start of incubation up to 12 hr. After this procedure, the separated tumor cells were incubated in the absence of PMN for various periods of time, and ^{3}H-UdR release into the supernatants was examined. When the tumor cells were separated from PMN at 3 hr after the start of incubation, a very low but significant increase of percent net lysis was observed in the following incubation. When the tumor cells separated from PMN at 6 hr or 12 hr of incubation were further cultured, a more prominent increase of percent net lysis was observed. Namely, the target tumor cells separated at 6 hr released ^{3}H-UdR at a level near that of the group in which target tumor cells were incubated with PMN during the whole assay time. Percent net lysis of the 12-hr separation group was more prominent, and almost the same as that of the group incubated with PMN during the whole assay time (Fig. 3). This result indicates that the time required for priming of the subsequently occurring cytolysis is 6 to 12 hr, and further interaction of target cells with PMN is not needed for the subsequent expression of cytolysis.

Inhibition of PMN Cytolysis by Heparin To further investigate the mechanisms of cytotoxicity by PMN pretreated with LPS WRT-7 sup, we examined the inhibitory effect on PMN cytolysis of radical oxygen scavengers, inhibitors of heme enzymes, and an inhibitor of neutral proteinases. As shown in Table II, none of the above-mentioned reagents in-

Table II. The Effect of Several Chemical Agents on Cytolysis Mediated by LPS WRT-7 Sup Treated PMN[a]

Inhibitor	(dose)	% Inhibition of cytolysis[b] E/T=50	E/T=25
Catalase	1000 U	−10.2	−8.0
SOD	400 U	−3.5	−0.5
Histidine	10m*M*	−3.7	2.2
Mannitol	10m*M*	−11.2	−7.8
Heparin	50 U	82.4[d]	58.3[d]
Aprotinin	5 U	0.0	ND[c]
NaN_3	0.5m*M*	−7.2	−1.1
KCN	0.5m*M*	−5.2	−8.0

a) Final dilution of sup=1/2. Percent cytolysis at E/T=50, 52.1%, E/T=25, 45.8%.
b) Percent inhibition of cytolysis was calculated by applying the following formula: (% inhibition of cytolysis) = 100× (% cytolysis without chemical agent − % cytolysis with chemical agent)/(% cytolysis without chemical agent).
c) Not done.
d) $P<0.01$.

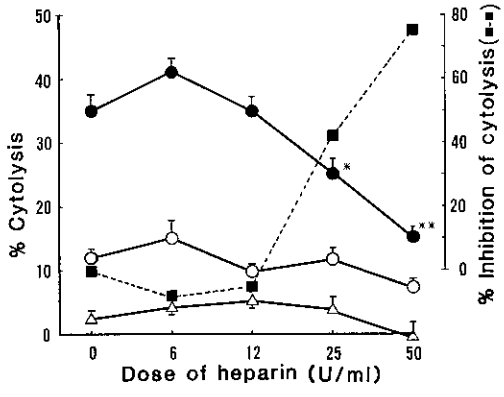

Fig. 4. Dose-response relationship for the inhibitory effect of heparin on cytolysis mediated by LPS WRT-7 sup-treated PMN. Percent inhibition of cytolysis was calculated by applying the formula shown in Table II. MEM was used as a control for estimating the effect of heparin on PMN. Final dilution of each sup=1/2, assay time=40 hr, E/T ratio=50:1, bars=SEM. LPS WRT-7 sup (●), LPS in c-IMDM (○), MEM (△), % inhibition of cytolysis (··· ■ ···). $^*P<0.05$, $^{**}P<0.01$.

hibited cytolysis at doses which had been reported to inhibit PMN cytotoxicity, which suggests that active oxygens such as superoxide, H_2O_2, hydroxyl radical and singlet

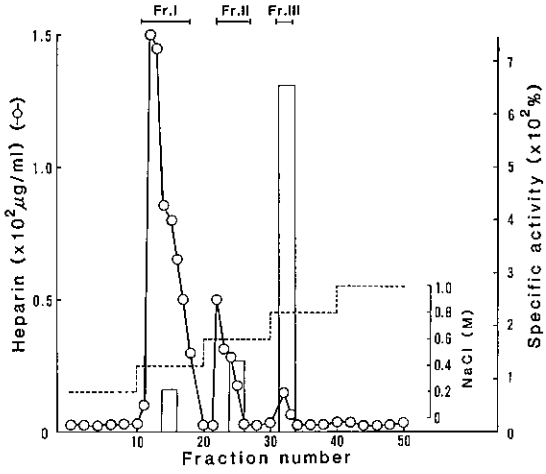

Fig. 5. The effect of heparin fractionated on a DEAE-Sephadex A-25 column on PMN-mediated cytolysis. Three peaks eluted from the DEAE-Sephadex A-25 column were collected (Fr. I, Fr. II, Fr. III); the concentrations of heparin in these fractions were 740 μg/ml, 480 μg/ml, and 120 μg/ml, respectively. These fractions were tested for inhibitory effect on cytolysis. Specific activity of heparin was expressed as percent inhibition of PMN cytolysis per milligram of heparin at the E/T ratio of 50:1 (□). Concentration of heparin (○), eluant molarity (----), percent lysis in the absence of heparin = 50.3%.

oxygen as well as the H_2O_2-myeloperoxidase-halide system or neutral protease are not the effector molecules in the PMN cytolysis observed in the present experiment. The only reagent showing an inhibitory effect was heparin. When various doses of heparin were used for the inhibition of PMN cytolysis, the inhibition rate was dependent on the dose of heparin used (Fig. 4).

To investigate the relationship between the negative charge of heparin and its capacity for inhibition of PMN cytolysis, heparin sodium solution was fractionated on a DEAE-Sephadex A-25 columu. Three major fractions were obtained by discontinuous changing of the eluant molarity, and each fraction was used for inhibition of PMN cytolysis. Fractions I and II, which were eluted earlier, showed a weak inhibition, while the later fraction (Fraction III) strongly inhibited PMN cytolysis (Fig. 5). This result suggests that the negative charge of heparin is a prerequisite for

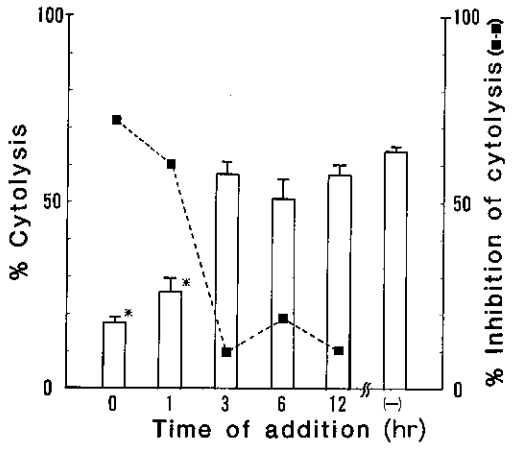

Fig. 6. Kinetics of inhibition by heparin of cytolysis mediated by LPS WRT-7 sup-treated PMN. Heparin (50 U/ml) was added to the mixture of target tumor cells and PMN at various points in time. Time in the figure is the time of addition of heparin after the start of the incubation. Percent cytolysis (□), percent inhibition of cytolysis (---■---). Final dilution of sup used=1/2, E/T ratio=50:1, bars=SEM. $^*P<0.01$.

the inhibition. To determine at which stage heparin inhibits PMN cytolysis, heparin was added to the reaction mixture of the target cells and PMN at various points in time after the start of PMN cytolysis assay. Heparin inhibited PMN cytolysis only when it was added between the start of assay and 1 hr thereafter (Fig. 6). After the pretreatment of PMN with various concentrations of heparin for various periods of time, the reaction mixtures were washed and then PMN cytolysis assay was started by addition of target RL♀-1 cells. No inhibition of PMN cytolysis was observed (data not shown), which suggests that inhibition of PMN cytolysis by heparin was not caused by a direct cytotoxic effect of heparin on PMN.

Discussion

In the present paper, we demonstrate that a signal required for cytolysis by cytokine-treated PMN appears at a relatively early phase of the interaction of PMN and target cells, and that heparin is an inhibitor of this PMN reaction. We obtained almost the same results concerning the above-mentioned two points using other sources of NAF such as supernatants from spleen cells incubated with a streptococcal preparation, OK - 432 (Hayashi, unpublished data). Thus, it may be stated that the above-mentioned phenomena are general features of PMN cytotoxicity induced by cytokine treatment.

The difference in the mode of PMN cytolysis mediated by active oxygens reported by many investigators and that induced by cytokine treatment as shown in the present experiment may be the same as the difference between the action mechanisms of bactericidal antibiotics and those of bacteristatic ones. The effector molecules responsible for PMN cytotoxicity induced by cytokines may be not as potent as active oxygens, and as a result the cytostatic effect on target tumor cells may be induced by the effector molecules, but they may not kill target cells promptly. However, when we consider the time required for the signaling of cytolysis, 6 to 12 hr of incubation was sufficient for the priming of cytolysis in the present experimental systems, and in that sense, the action of PMN was already finished in the early stages of the reaction.

As to the mechanisms of inhibition of PMN cytolysis by heparin, Clark *et al.* have demonstrated that cationic proteins purified from human neutrophil granules exert a cytotoxic effect on mammalian tumor cells, and that this effect is inhibited by heparin.[20] In contrast to our present results, they found that cationic proteins killed tumor cells in a short time, such as two to three hours. The discrepancy may be ascribed to the difference in the system used. The medium used in their system was a simple phosphate-buffered saline, and they used purified cationic proteins but not the live neutrophils we used. Furthermore, it has been demonstrated that heparin inhibits killing of *Trypanosoma cruzi* by eosinophils.[21, 22] In this case, heparin action is ascribed to the inhibition of cytotoxic major basic protein activity in eosinophils. We have recently demonstrated inhibitory activity of heparin on natural killer cell-mediated cytotoxicity.[23] Taken together, although the mechanisms of cytotoxicity by PMN activated with cytokines are far from clear, certain cationic proteins in neutrophils may be involved in the PMN cytotoxicity observed in

the present study. The validity of this speculation may be strengthened by the result that the heparin fraction possessing strong negative charges showed a prominent inhibitory activity. Moreover, it has been reported very recently that low-molecular cationic proteins, defensins, of human and rabbit PMN show tumor cytotoxicity.[24] This molecule may be a candidate for the effector molecule in cytokine-activated PMN cytotoxicity.

The result that heparin inhibited PMN cytolysis only when it was added early in the assay may support the assumption that signaling for subsequent cytolysis is finished in the early phase of the reaction. However, heparin failed to inhibit subsequent cytolysis when it was added at 3 hr after the start of incubation, but separation of target cells from PMN at this point did not give sufficient ^{3}H-UdR release from target cells during the following incubation. These two results may suggest that heparin acts in the early stage in the signaling, and it is required in a further step that may not be inhibited by heparin for accomplishing the priming of cytolysis.

As to the role of LPS in tumor cytotoxicity in the present experiments, although a major part of LPS in LPS WRT-7 sup was inactivated by polymyxin B, it seems likely that a small dose of LPS remained and played a certain role in the activation of PMN, inasmuch as it has been demonstrated that LPS shows a priming effect on the subsequent activation of PMN.[25] However, this possibility may not affect the above conclusion, since as we mentioned before, the same result was obtained in the experimental system of an LPS-free condition.

Nonoxidative mechanisms of PMN cytotoxicity have also been demonstrated by other investigators.[26, 27] It seems out of the question that oxidative mechanisms play an important role in tumor cytotoxicity by PMN. The above-mentioned and our present results suggest the existence of nonoxidative mechanisms in certain systems, and the present experimental system may provide a model for studying the mechanisms of non-oxygen dependent PMN cytotoxicity.

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