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Enhancement of Polymorphonuclear Leukocyte-mediated Tumor Cytotoxicity by Serum Factor(s)

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It has been reported that β-1,3-D-glucan isolated from *Alcaligenes faecalis* (TAK) promoted tumor cytolysis by mouse polymorphonuclear leukocytes (PMN). We investigated the effect of serum on mouse PMN tumor cytolysis induced by TAK and other PMN stimulators. Addition of fetal calf serum (FCS) to the cytolysis assay enhanced tumor cytolysis by PMN in a dose-dependent manner. Sera obtained from horses, mice, and rats were also effective enhancers of PMN tumor cytolysis. When FCS was added after the assay was under way, the enhancing effect decreased proportionally to the time elapsed. The enhancing activity was detected over a broad range of fractions with a peak at 170 kD by fractionation on a Superose 6 column. The responsible factor(s) in serum was stable after treatment at 60°C, 30 min or after lowering the pH to 2. Mouse PMN stimulated with TAK increased production of hydrogen peroxide in the presence of FCS.

Key words: Polymorphonuclear leukocytes — Tumor cytotoxicity — Serum factor(s)

Among effector cells involved in cell-mediated tumor cytotoxicity, little attention has been paid to polymorphonuclear leukocytes (PMN)[4] compared with others, such as cytotoxic T cells, natural killer cells, and macrophages. Recently, however, several studies have shown that PMN are potential effector cells, especially when tumor-bearing hosts are treated with biological response modifiers (BRM).[1-3] Studies of the mechanisms involved in tumor cytotoxicity by PMN stimulated with BRM have been undertaken very recently,[4-6] but the details, including regulation of PMN tumor cytotoxicity, remain to be resolved.

In the course of our studies, we noticed that serum exerted a modulatory effect on PMN tumor cytotoxicity. Several immunoregulating factors have been found in serum, some of which have been purified. Of these, the majority affect the afferent loop of the immune response, and relatively little attention has been focused on the effects of serum on the effector phase. The role played by serum factors on functions of PMN has been elucidated in several different systems. Alpha 1-acid glycoprotein (AAG) was reported to inhibit phagocytosis of *Staphylococcus aureus* and *Escherichia coli*,[7] aggregation, and superoxide generation by PMN.[8] The sera of cancer patients were reported to inhibit PMN-mediated cytotoxicity,[9] leukotaxis[10] and expression of the Fc receptor on neutrophils.[11]

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[4] Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; TAK, linear β-1,3-D-glucan from *Alcaligenes faecalis*; FCS, fetal calf serum; MEM, Eagle's minimum essential medium; PEC, peritoneal exudate cells; E/T, effector-to-target ratio; [^{3}H]Urd, tritiated uridine.

In contrast to these negative regulatory effects of serum on PMN function, a positive effect has also been reported. Sedgwick *et al.* demonstrated that plasma or sera from animals with carrageenan pleurisy enhanced the ability of PMN to adhere to vascular endothelial cells.[12] We demonstrate in the present paper, that tumor cytotoxicity by mouse PMN induced by a linear β-1,3-D-glucan[1] was significantly enhanced by the sera of all the species examined.

MATERIALS AND METHODS

Mice and tumor cells C3H/He, C57BL/6, and ddY mice were purchased from Shizuoka Animal Co., Ltd., Shizuoka. Tumor cells used as target cells in cytolysis assay were as follows: RL♀1; an X-irradiation-induced lymphoma of BALB/c, MM46; a spontaneous breast cancer of C3H/He, and YAC-1; a Moloney leukemia virus-induced lymphoma of A/Sn. These tumor cells were maintained in tissue culture. We used RL♀1 as the target tumor cell line unless otherwise stated.

Culture media Iscove's modified Dulbecco's medium (IMDM, Gibco Lab., Chagrin Falls, Ohio) was supplemented with 0.5 g/liter bovine serum albumin (Sigma, St. Louis, Mo.), 5 mg/liter transferrin (Sigma), 100 mg/liter soybean lipid (Sigma) according to Iscove and Melchers[13] and 5 mg/liter insulin (Sigma). This medium was used as a serum-free complete medium (SFCM). Heat-inactivated fetal calf serum (FCS) (Gibco Lab.), or heat-inactivated sera from horses, rats, and mice were added to SFCM. RPMI 1640 (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% FCS, 100 μg of streptomycin, 100 U/ml, penicillin-G, 2 mg/ml $NaHCO_3$

and 25 m*M* HEPES (Dojin Laboratory Co., Ltd., Kumamoto) was used for maintenance of target cells.

Preparation of PMN Four milliliters of 3% proteose peptone (Difco, Detroit, Mich.) was injected intraperitoneally into ddY mice, and a booster injection of 4 ml of proteose peptone was administered 12 h later. At 2–3 h after the booster injection, peritoneal exudate cells (PEC) were obtained by peritoneal lavage, and used as PMN-rich cell suspensions. PEC were washed twice with Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co.) and suspended in SFCM. The quantity of PMN in PEC was 80 to 85% as determined by May-Giemsa staining. Further purification of PMN from PEC was carried out as described by Hanson *et al.*[14] and Goto *et al.*[15] Briefly, PEC were placed on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden), and centrifuged at 400*g* for 1 h at room temperature. The Percoll gradient consisted of layers of 70%, 65%, 60%, 50% and 30%. The cell suspensions in the bottom layer contained more than 98% PMN. We used the above-mentioned PEC as an effector source unless otherwise stated.

Cytolysis assay The cytolysis assay was performed with a 96-well microplate (Sumitomo Multiplate 96F II, Sumitomo Co., Tokyo) as already reported elsewhere.[16] Target cells were labeled with 5 μCi/ml tritiated uridine ([^{3}H]Urd) (Amersham Japan, Tokyo) at 37°C, in 5% CO_2-95% air, for 3 h. The labeled target cells were washed twice with MEM, and then suspended in SFCM. Twenty thousand labeled target cells per well were incubated with various numbers of PMN in the presence or absence of linear β-1,3-D-glucan (TAK) and other PMN stimulators usually for 16 to 18 h at 37°C and in the presence of 95% air and 5% CO_2. We mainly used RL♀1 as target cells because this tumor line had the highest sensitivity to PMN cytotoxicity. The culture medium used for the cytolysis assay was SFCM in the presence or absence of sera from various species. The total volume of the reaction mixture was 200 μl. After incubation, the plates were centrifuged at 150*g* for 5 min, and an aliquot of the supernatant (100 μl) was obtained. Supernatants from each experiment were mixed with 1.5 ml of ACS-II (Amersham Japan) solution in a counting vial, and radioactivity was determined with a liquid scintillation counter (Rackbeta 1216, LKB Wallac, Turku, Finland). Percent specific lysis was calculated using the following formula:

$$\%\text{lysis}=\frac{\text{cpm of experimental group}-\text{cpm of spontaneous release}}{\text{cpm of maximum release}-\text{cpm of spontaneorelease}}\times 100.$$

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

Hydrogen peroxide assay Generation of H_2O_2 by PMN was measured as described by Pick and Keisari.[17] One milliliter of mouse PMN (2.5×10^6/ml), TAK (1 mg/ml) and FCS (from 0% to 10%) was incubated at 37°C for 1 h in phenol red solution (PRS). PRS contained 140 m*M* NaCl, 10 m*M* potassium phosphate buffer, pH 7.0, 5.5 m*M* dextrose, 0.28 m*M* phenol red and 8.5 U/ml of horseradish peroxidase. After incubation, the reaction mixture was centrifuged at 400*g* for 5 min, and the whole supernatant was transferred to another tube. After addition of 30 μl of 1 *N* NaOH, absorbance at 610 nm was measured with an electron photometer (Hitachi 100-10). A standard curve was generated with 1–25 μM H_2O_2 solution using PRS.

Gel filtration FCS was fractionated using a fast protein liquid chromatography apparatus (FPLC, Pharmacia Fine Chemicals). One milliliter of FCS was passed through an HA filter and applied at 0.5 ml/min to a Superose 6 column (16×500 mm) equilibrated with phosphate buffer (25 m*M*, pH 6.7). Fractions of 2.5 ml were collected. The procedure was repeated 15 times, and samples obtained from the same fraction number were pooled. After dialysis and lyophilization, the sample was suspended in 1 ml of SFCM and used in the cytolysis assay. The protein concentration of each pooled fraction was determined with a Bio-Rad protein assay kit (Bio-Rad Lab., Richmond, Ca.).

Purification of the mouse IgG Mouse IgG was purified by FPLC from ascites fluid containing monoclonal antibody (MoAb), AZPO-8. The procedure for establishment of the MoAb (AZPO-8) and the nature of the MoAb were described in a previous paper.[18] The subclass of AZPO-8 was IgG_1. The procedure for purification of IgG was as described in a previous paper.[19]

Tumor cytolysis by PMN stimulated with lymphokines As lymphokine sources for stimulation of PMN, we used culture supernatants from mouse spleen cells stimulated with a streptococcal preparation, OK-432 (OK sup)[20] and those from Jurkat lymphoma (Jurkat sup).[21] PMN suspensions were treated with these supernatants for 3 h at 37°C in the presence of 0 to 20% FCS. The method for PMN treatment was described in detail elsewhere.[22] After being washed twice with MEM, the treated PMN was mixed with [^{3}H]Urd-labeled target cells, and the reaction mixtures were incubated for 24 h at 37°C in the presence of FCS at the same concentrations as used for pretreatment of PMN with the culture supernatants. At the end of the incubation, the radioactivity of the supernatants was measured.

Reagents Linear β-1,3-D-glucan isolated from *Alcaligenes faecalis* var. IFO 13140 (TAK) was kindly supplied by Takeda Pharmaceutical Co. (Osaka). BCG was

purchased from Nippon BCG Co. (Tokyo). Zymosan A, horseradish peroxidase, bovine catalase, superoxide dismutase, human fibronectin, anti-human fibronectin, bovine thyroglobulin, bovine γ-globulin, bovine serum albumin, and phenol red were purchased from Sigma Chemical Co.

Statistical studies Statistical analyses were performed with Student's *t* test.

RESULTS

Enhancement of TAK-induced PMN tumor cytolysis by FCS The PMN-rich suspension from C3H/He mice showed a strong cytotoxicity to MM46 cells in the presence of TAK as already reported by Morikawa *et al.*,[1,4] provided that the assay medium contained 5% FCS. PMN, however, exhibited only a weak cytotoxicity even in the presence of TAK when the assay medium did not contain FCS (Fig. 1). The presence or absence of FCS in the assay medium did not affect spontaneous release of [^{3}H]Urd from target cells, nor did the addition of TAK. Augmentation of cytolysis was dependent on the concentration of FCS added up to 20% (Fig. 2). When we added higher concentrations of FCS such as 50% and 75%, almost the same or slightly higher cytolysis was observed compared with that of the 20% FCS group. Similar results were obtained when assay times were shortened to 6–8 h (data not shown), or when target cells other than RL♀1 (MM46, YAC-1) were used. Values of percent specific cytolysis of MM46 and YAC-1 at an E/T ratio of 12/1 were 62.5±1.2 and 13.4±0.3, respectively, in the presence of FCS, and 5.3±0.7 and −3.9±0.1, respectively, in the absence of FCS. Since PMN-rich suspensions contained other cells, the effect of serum on the cytotoxicity was examined using purified PMN obtained as described in "Materials and Methods." Identical results were obtained with purified PMN (Fig. 3), that is, cytotoxicity induced by TAK was significantly enhanced by addition of FCS to the assay medium. This indicates that serum efficacy could not be ascribed to

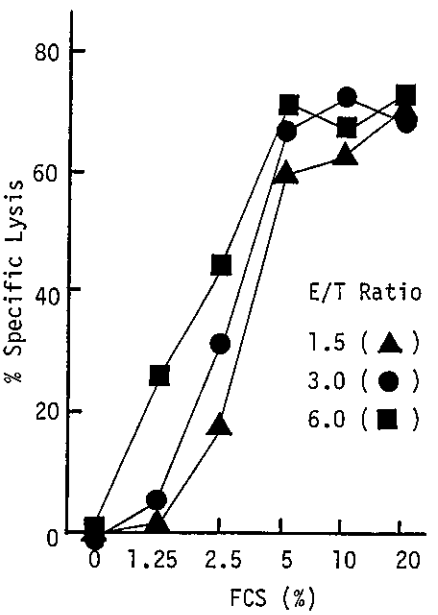

Fig. 2. Dose-response relation of FCS on PMN cytotoxicity induced by TAK. TAK: 500 μg/ml. Incubation time: 18 h. Spontaneous releases (%) of each group were as follows (% FCS is noted in parenthesis): 23.2±0.1 (0), 21.6±1.8 (1.25), 22.4±0.8 (2.5), 21.7±0.6 (5.0), 22.7±0.9 (10.0), 21.4±1.0 (20.0). Nonstimulated PMN showed less than 5% tumor cytotoxicity.

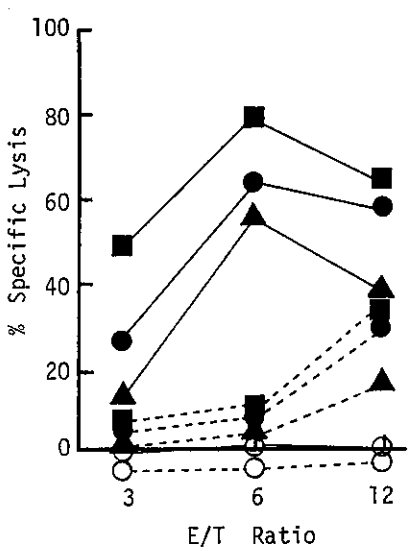

Fig. 1. Enhancement by FCS of PMN cytotoxicity induced by TAK. Straight lines (——) show PMN cytotoxicity in the presence of 20% FCS, and dashed lines (------) show that in the absence of FCS. Incubation time: 16 h. All points in FCS (+) groups were significantly higher than in FCS (−) ones when PMN were stimulated with TAK ($P<0.01$).

Conc. of TAK (μg/ml)	Symbol	Spontaneous release (%) Percent of FCS 0	20
1,000	(■)	18.3±0.2	13.7±0.1
500	(●)	19.2±0.5	14.2±0.4
250	(▲)	18.9±1.7	15.9±0.1
0	(○)	21.6±1.4	14.3±0.5

other cells contaminating the PMN suspension. These results suggest that FCS contains certain factor(s) that augment the cytotoxicity of PMN stimulated with TAK.

In order to discover whether the above-mentioned phenomena are a general feature of PMN cytotoxicity, we examined the effect of serum on PMN cytotoxicity induced by stimulators other than TAK. Fig. 4 shows

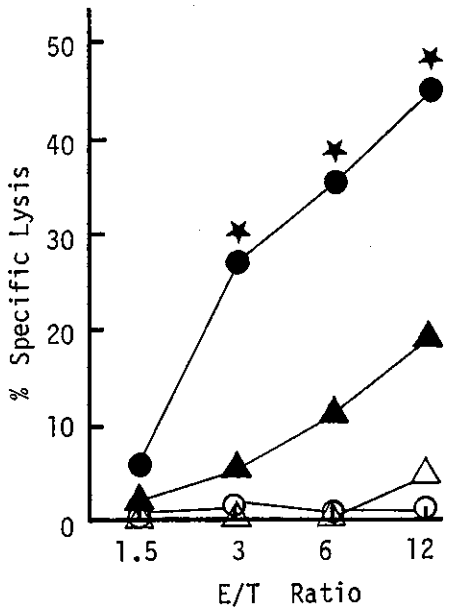

Fig. 3. Enhancement by FCS of TAK-induced tumor cytolysis by highly purified PMN. ★: Significantly higher than FCS(−), TAK(+) group ($P<0.01$). Incubation time: 16 h.

Symbol	TAK(500 μg/ml)	FCS (20%)	Spontaneous release (%)
(●)	(+)	(+)	17.5±1.1
(▲)	(+)	(−)	15.5±0.5
(○)	(−)	(+)	16.6±0.5
(△)	(−)	(−)	16.4±0.5

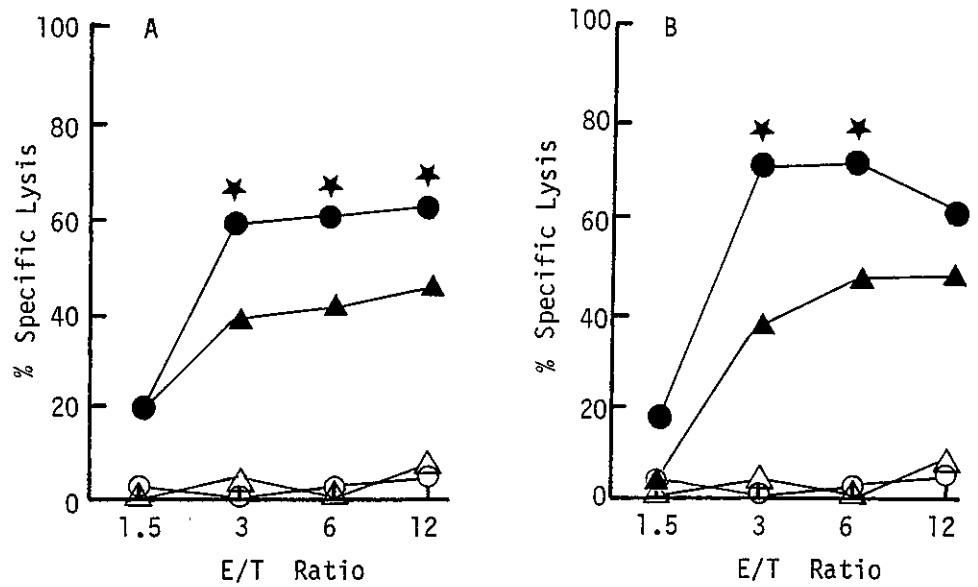

Fig. 4. Enhancement by FCS of PMN cytotoxicity induced by zymosan A and BCG. ★: Significantly different from FCS(−) group ($P<0.01$). Incubation time: 16 h.

	Symbol	PMN stimulator (500 μg/ml)	FCS (20%)	Spontaneous release (%)
A	(●)	Zymosan A	(+)	11.1±0.3
	(▲)	Zymosan A	(−)	13.3±0.9
B	(●)	BCG	(+)	10.4±0.2
	(▲)	BCG	(−)	12.1±0.1
A and B	(○)	(−)	(+)	12.3±0.2
	(△)	(−)	(−)	13.5±0.3

that PMN cytolysis induced by zymosan A and BCG was also enhanced by the addition of FCS, although not so dramatically as with TAK.

Enhancement of TAK-induced PMN cytolysis by sera from several species To investigate whether or not the phenomena observed were restricted to FCS, the sera from rats, mice, or horses were added to the assay medium and PMN cytolysis induced by TAK was examined. As shown in Fig. 5, all the sera studied augmented the cytotoxicity, although the degree of enhancement differed from species to species.

Mechanisms of PMN cytotoxicity augmentation by serum factor(s) To determine the stage at which serum factor(s) enhanced PMN cytotoxicity, a final concentration of 20% FCS was added to the reaction mixtures of PMN, TAK, and labeled cells at various times after the start of the assay. PMN cytotoxicity was most enhanced when FCS was added during the first 30 min of incubation. Augmentation activity gradually decreased when FCS was added later in the assay (Fig. 6). To test whether the serum factor(s) which enhanced PMN cytotoxicity were bound firmly to PMN, the following experiment was performed. A PMN suspension was incubated for 3 h at 37°C under 5% CO_2 and 95% air in the presence or absence of 20% FCS. After two washings with MEM, the PMNs were suspended in SFCM and treated with TAK and target cells in 0% or 20% FCS. Even when PMN had been pretreated with 20% FCS, they did not show significant cytolysis if the assay was performed in the absence of FCS (Group (+)⟶(−)). The group pretreated with 0% FCS showed significant cytolysis in the presence of 20% FCS (Group (−)⟶(+)) (Table I). These results suggest that the enhancing factor(s) do not bind firmly to PMN, and that enhancement of PMN cytotoxicity by serum factor(s) is seen only when these factors are present in the assay reaction mixture. It has been reported that H_2O_2 is the effector molecule in PMN cytotoxicity mediated by TAK, and

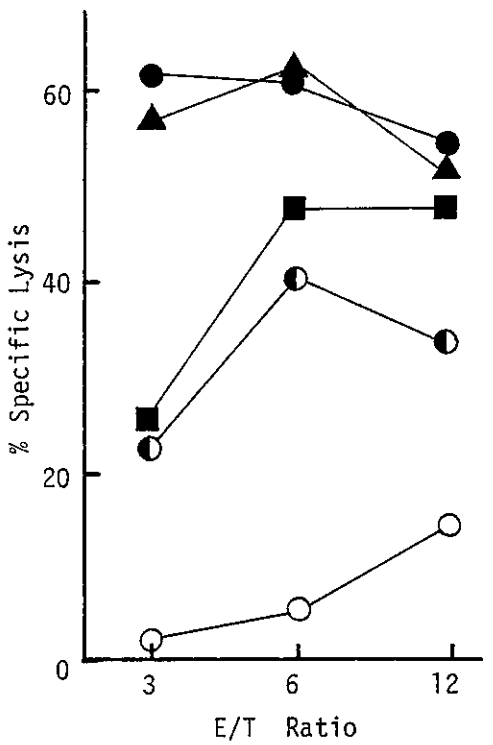

Fig. 5. Enhancement of TAK-induced PMN cytotoxicity by serum obtained from various species. TAK: 500 μg/ml. Incubation time: 18 h. Sera: 20%. All points of percent specific cytolysis in the presence of sera from various species were significantly higher than those of a serum-free control ($P<0.01$). Nonstimulated PMN showed less than 5% tumor cytotoxicity.

Symbol	Source of sera	Spontaneous release (%)
(▲)	Horse	14.2±0.2
(■)	Fetal calf	15.0±0.4
(●)	Rat	15.5±0.4
(◐)	Mouse	20.9±0.7
(○)	(Serum free)	19.1±0.5

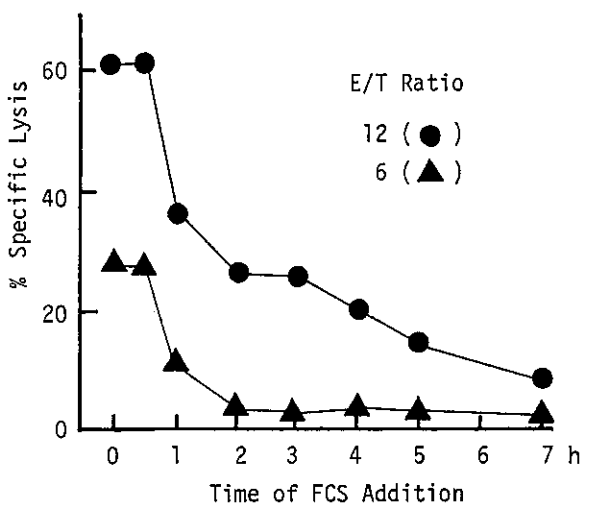

Fig. 6. Effects on TAK-induced PMN cytolysis of FCS added to the assay mixture at various intervals after the start of the assay. Fifty μl of 80% FCS was added at various times during the assay at a final concentration of 20%. Spontaneous releases (%) of each group were as follows (the time (hour) of addition of FCS is noted in parenthesis): 17.5±0.4 (0), 18.0±0.6 (0.5), 17.0±0.6 (1.0), 18.7±0.4 (2.0), 19.1±0.7 (3.0), 18.4±0.3 (4.0), 18.9±0.4 (5.0), 16.9±0.5 (7.0). TAK: 500 μg/ml. Incubation time: 7 h. Nonstimulated PMN showed less than 5% tumor cytotoxicity.

Table I. FCS Requirement in the Cytolysis Assay System for the Expression of FCS Enhancement of TAK-induced PMN Cytolysis

E/T ratio	FCS (20%) in the pretreatment		FCS (20%) in the cytolysis assay	Specific lysis (%)
6	(−)	⟶	(−)	10.6±0.7
	(−)	⟶	(+)	25.7±0.4
	(+)	⟶	(−)	5.7±0.1
	(+)	⟶	(+)	25.9±1.9
12	(−)	⟶	(−)	15.7±1.4
	(−)	⟶	(+)	42.5±0.6
	(+)	⟶	(−)	21.7±0.1
	(+)	⟶	(+)	37.0±0.2

Spontaneous release (%): 22.0±0.8 (−), 18.2±0.2 (+). Nonstimulated PMN showed less than 5% tumor cytotoxicity.

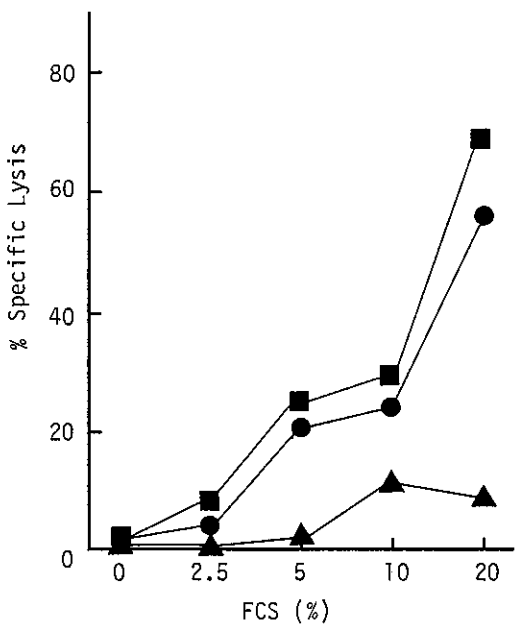

Fig. 7. Effects of catalase and superoxide dismutase on the enhancement by FCS of PMN cytotoxicity induced by TAK. TAK: 500 μg/ml. Incubation time: 18 h. E/T ratio: 12. Catalase (500 U/ml) (▲), superoxide dismutase (300 U/ml) (■), none (●). Values of spontaneous release (%) of each group were as follows (% FCS is noted in parenthesis): 25.2±0.3 (0), 23.5±1.3 (2.5), 22.1±1.0 (5.0), 20.2±0.8 (10), 20.0±0.6 (20). Nonstimulated PMN showed less than 5% tumor cytotoxicity.

Table II. Enhancement of H_2O_2 Release by PMN Stimulated with TAK in the Presence of FCS

Percent of FCS	H_2O_2 release (nmol/2.5×10⁶ PMN/60 min) Exp. 1		Exp. 2	
	TAK (500 μg/ml)			
	(−)	(+)	(−)	(+)
0	2.4	3.6	11.4	14.4
1.25	0	4.2	9.2	21.0
2.5	0	10.2	9.6	22.8
5.0	0	24.8	14.4	27.0
10.0	0	28.8	15.0	30.0

Details of the H_2O_2 assay are given in "Materials and Methods."

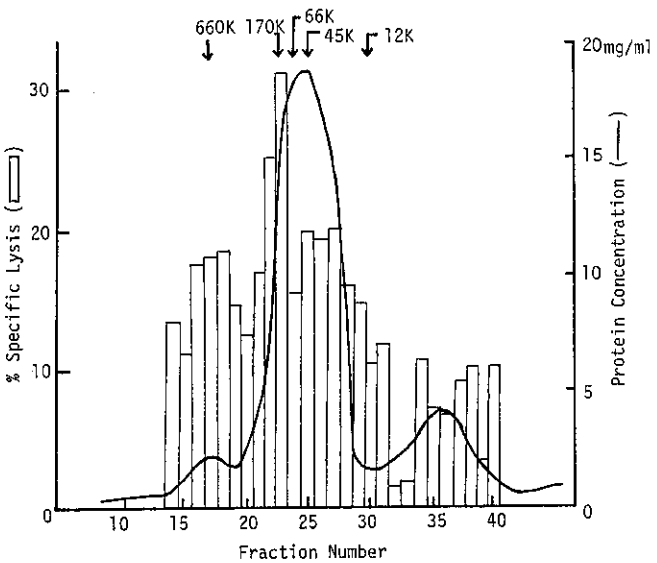

Fig. 8. Enhancement of TAK-induced PMN cytolysis by FCS fractionated on Superose 6. FCS was fractionated by FPLC as described in "Materials and Methods." Values of specific lysis in the presence and absence of 20% nonfractionated FCS were 32.7±0.2, and 4.1±0.2, respectively. Protein concentration is indicated at 1/8 final dilution. Protein concentration of 20% FCS was 28 mg/ml. TAK: 500 μg/ml. Incubation time: 16 h. E/T ratio: 6. Nonstimulated PMN showed less than 5% tumor cytotoxicity.

that PMN cytotoxicity induced by BCG and zymosan A is partially dependent on H_2O_2.[4] In order to investigate whether an effector molecule involved in the PMN cytolysis augmented by the serum factor(s) is H_2O_2 or not, catalase or superoxide dismutase was added to the reaction mixtures of the target cells, PMN suspensions, TAK, and graded concentrations of FCS. Catalase almost completely inhibited the enhancement by FCS of TAK-induced PMN cytotoxicity, but superoxide dismutase did not (Fig. 7). Furthermore, we studied whether or not H_2O_2 release from TAK-stimulated PMN is enhanced by addition of FCS to the medium. In doing so,

we employed the phenol red method instead of the scopoletin horseradish peroxidase method to avoid the quenching effect of FCS. Table II shows that H_2O_2 production by TAK-stimulated PMN was significantly higher in the presence of FCS than in its absence.

Attempts to characterize serum factor(s) that enhance PMN tumor cytotoxicity Each fraction of FCS (No. 14–40) eluted from Superose 6 was added to the assay at final concentrations of 1/4, 1/8, and 1/16, and its effect on TAK-induced PMN cytotoxicity was examined. Cytolysis-enhancing activity was detected over a broad fractionation area with the peak at 170 kD (representative data with a sample of at 1/8 final dilution are shown in Fig. 8). To determine if augmentation is ascribable to a nutritional requirement, bovine serum albumin (2.5 mg/ml) was added to the assay. We did not detect any cytotoxicity-enhancing activity. We also examined the effect of fibronectin (125 μg/ml) on PMN cytolysis since it has been reported that fibronectin augments PMN adhesion. No enhancing effect was noted. Furthermore, anti-fibronectin serum did not inhibit PMN cytolysis performed in the presence of 10% FCS. Treatment of FCS at 56°C for 30 min, or 60°C for 30 min, hardly affected the enhancing activity of FCS, although a 100°C treatment for 10 min abolished its activity. Lowering the pH to 2 did not affect the enhancing activity of FCS (Fig. 9). Purified mouse IgG enhanced PMN cytotoxicity in a dose-dependent manner (Fig. 10). To determine whether contaminating LPS in FCS could be a causative agent for augmentation of PMN cytolysis, polymyxin B was added to the assay. We found that this addition did not alter the augmentation effect of FCS (data not shown).

The effect of FCS on tumor cytolysis by PMN stimulated with lymphokines In contrast to the results using TAK as a stimulant, PMN cytolysis induced by OK sup and Jurkat sup was reduced in proportion to the concentra-

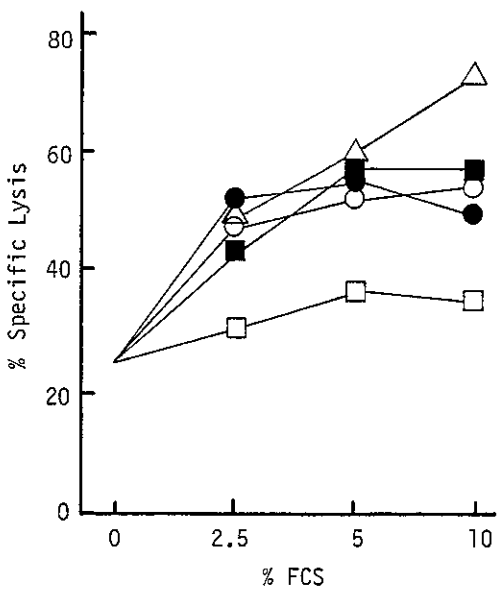

Fig. 9. Physicochemical stability of cytolysis-enhancing factors in FCS. FCS was treated at 56°C for 30 min (○), 60°C for 30 min (●), 100°C for 10 min (□). FCS was kept at pH 2 for 2 h, and then restored to pH 7 (■). Untreated FCS (△). Specific lysis of groups treated at 56°C for 30 min, 60°C for 30 min, and at pH 2 was not significantly different from that of the non-treated FCS group. That of the group treated at 100 °C for 10 min was significantly lower than that of the non-treated FCS group ($P<0.01$). E/T ratio: 6. TAK: 500 μg/ml. Incubation time: 16 h. Nonstimulated PMN showed less than 5% tumor cytotoxicity.

Treatment of FCS	Spontaneous release (%)		
	Percent of FCS		
	2.5	5.0	10.0
56°C 30 min	17.8±1.2	15.9±1.2	14.3±0.2
60°C 30 min	18.5±0.1	18.8±0.9	18.0±0.7
100°C 10 min	19.1±0.1	17.2±0.7	18.4±1.2
pH 2	18.9±1.2	17.5±0.5	17.7±0.5
None	17.2±0.4	19.0±1.4	18.4±1.4
No FCS		19.7±0.4	

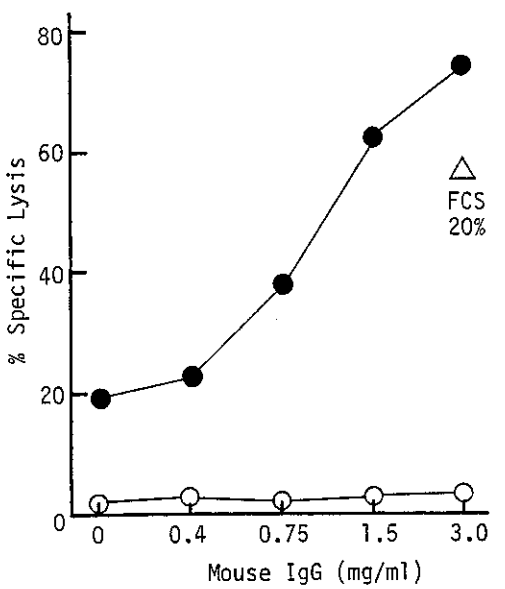

Fig. 10. Enhancement of TAK-induced PMN cytolysis by purified mouse IgG. Mouse IgG_1 was purified from a monoclonal antibody as described in "Materials and Methods." Fifty μl of serially diluted IgG_1 was added to the mixture of PMN and TAK (500 μg/ml) (●) or without TAK (○). Spontaneous releases of each group were as follows (dose of IgG_1 is noted in parenthesis): 24.6±0.1 (0), 34.8±0.1 (0.4), 32.4±1.8 (0.75), 29.5±1.6 (1.5), 31.2±1.8 (3.0). 21.6±0.1 (20% FCS). Incubation time: 17 h. E/T ratio: 6.

Table III. The Effect of FCS on Tumor Cytolysis by PMN Stimulated with Lymphokines

Percent of FCS	Specific lysis (%)		
	PMN stimulators		
	TAK	OK sup	Jurkat sup
0	2.4±0.2	30.2±0.5	42.2±0.9
2.5	9.8±0.1	23.0±0.4	32.2±0.8
5.0	20.2±0.3	14.1±0.4	29.3±0.4
10.0	25.3±0.2	11.0±0.3	24.1±0.2
20.0	55.2±0.4	9.2±0.1	16.1±0.1

Spontaneous releases (%) of each group were as follows (% of FCS is noted in parenthesis): 22.9±2.8 (0), 18.0±1.8 (2.5), 18.6±1.6 (5.0), 18.1±3.2 (10.0), 17.8±1.7 (20). Non-stimulated PMN showed less than 5% tumor cytotoxicity. PMN was pretreated with 1/2-diluted OK sup or 1/2-diluted Jurkat sup. TAK: 500 μg/ml, 18 h incubation, E/T: 6. OK sup and Jurkat sup: 24 h incubation, E/T: 50.

tion of FCS (Table III). This result shows that the effect of FCS on PMN cytotoxicity differs depending on the PMN stimulants used.

DISCUSSION

We have shown in this paper that certain serum factor(s) enhance PMN-mediated tumor cytolysis induced by a linear β-1,3-D-glucan as well as by other PMN stimulators. Serum factor(s) are not obligatory for cytolysis by PMN, but they do enhance the reaction. Based on our experiments and previous work by Morikawa *et al.*,[1] it is clear that significant cytolysis by PMN occurs even in the absence of FCS.

Serum factors that augment PMN and macrophage action have been reported by several workers. Chapman and Hibbs[23] reported that macrophage tumoricidal capability was enhanced by a component of normal serum, and that enhancement was maintained even after serum components were washed out of the assay system. Furthermore, the augmenting factor(s) were rather heat-labile. We feel, therefore, that their factor(s) are different from those observed in our experiments. In a non-tumor cytolysis system, Gerberick *et al.*[24] found that serum factor(s) were required for reactive oxygen intermediate release by rabbit alveolar macrophages. Leonard and Skeel[25] reported a "macrophage-stimulating protein" which enhanced chemotaxis. Johnston *et al.* demonstrated increased superoxide production by macrophages after culture in FCS.[26] Furthermore, Sedgwick *et al.*[12] reported that plasma from animals with carrageenan pleurisy enhanced the adhesive activity of PMN. We have now added another aspect to the augmentation of PMN activity by serum factors.

Although the precise mechanisms of serum factor augmentation of PMN cytotoxicity are still nuclear, enhancement of H_2O_2 production by stimulated PMN in the presence of serum (Table II) may be at least partially responsible. Morikawa *et al.* reported that H_2O_2 was the effector molecule in mouse PMN cytotoxicity induced by TAK.[4] Furthermore, enhancement by serum factor(s) of TAK-induced cytotoxicity was almost completely inhibited by catalase (Fig. 7). Our preliminary experiments showed a partial inhibition by catalase of zymosan A- or BCG-induced PMN cytotoxicity (Araki, unpublished results). Almost complete as well as partial dependency on FCS of PMN cytotoxicity induced by TAK and zymosan A or BCG, respectively (Fig. 4), may be explained by a difference in the role of H_2O_2 as the effector in PMN cytotoxicity induced by these stimulators. As already discussed above, serum factor(s) are required for release of active oxygen by alveolar macrophages.[24] However, there are some differences between their experiments and ours in terms of the effect of removal of FCS from the assay system and the time required for augmentation.

The results that binding between target cells and PMN was not enhanced by addition of FCS and that anti-fibronectin antibody did not affect the phenomena (Araki, unpublished results), suggest that a possible increased binding between target and effector cells is not a prerequisite for the observed phenomena. Enhancement of release of cytotoxic factor(s) other than H_2O_2 from PMN is not also a candidate, inasmuch as we have never found such factor(s) in the supernatants of PMN in our systems (Sendo, unpublished results). Serum or plasma factors such as complement components, coagulation factors[27] and fibronectin[28, 29] that have been reported to augment activities of PMN and macrophages may not be involved in the phenomena observed in the present experiments. LPS is also not a likely candidate. Nutrition or growth factors such as serum albumin, insulin, transferrin or platelet-derived growth factor (PDGF) can be ruled out, since the former three substances were present in the medium used, and PDGF-free human serum augmented PMN cytotoxicity (Araki, unpublished results). Thus, the mechanism of enhancement by serum factors of TAK-induced PMN cytotoxicity is still quite unclear, as is the situation regarding the effect of serum factor(s) on the function of neutrophils and macrophages.[23-26] Further investigation of the precise mechanisms involved in serum enhancement is required.

The activity augmenting PMN tumor cytolysis in FCS was observed over a broad fractionation range on Superose 6, indicating that the factor(s) are heterogeneous. This is similar to the previous result on the augmenting effect of serum factor(s) on reactive oxygen intermediate release by rabbit alveolar macrophages. In that case the

activity was found over a broad fractionation range on Sephacryl S-200 column with a peak at 30–50 kD. Other results have been obtained in serum factor augmentation of prostaglandin production by alveolar macrophages. Prostaglandin-inducing activity was eluted over a broad fractionation range with a peak at 150 kD. IgG on its own showed augmenting activity.[30] The result that the molecular size of the peak activity in our experiment was also approximately 170 kD (Fig. 8) led us to examine the effect of purified monoclonal mouse IgG. The purified IgG actually augmented TAK-induced PMN cytotoxicity (Fig. 10). Considering all these results, it is likely that the effect of serum factors on certain PMN and macrophage functions as observed in our experiment may not be ascribed to a single molecule. The activity may rather be derived from a common nature of different molecules. Otherwise, we are unable to explain the result that purified IgG and other molecules in the serum are equally effective in affecting the activity of phagocytes, as observed in our experiment, and in the production of prostaglandin by macrophages.[30] More precise biochemical analysis on the nature of the factors may resolve these problems.

We previously reported a decrease in PMN cytotoxicity by FCS induced by lymphokines obtained from several sources. We found it to be inhibited by FCS in a dose-dependent manner.[31] We also showed in this paper that addition of FCS inhibited tumor cytotoxicity by PMN stimulated with culture supernatants from mouse spleen cells incubated with a streptococcal preparation, OK-432, and those from Jurkat lymphoma (Table III). Although the effector molecules in lymphokine-induced PMN cytotoxicity have not been determined, none of the active oxygen scavengers inhibited cytotoxicity, and only heparin inhibited the reaction.[6] Lichtenstein *et al.* also demonstrated that cytotoxicity by defensin, a cationic cytotoxic peptide in azul granules of PMN, is inhibited by serum factor(s).[32] These results suggest that under certain conditions the serum factors augment PMN tumor cytotoxicity, but in other situations they show an opposite effect. In conclusion, serum components may play an important regulatory role in the expression of tumor cytotoxicity by PMN in certain systems.

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