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Antitumor Effect of *Streptococcus pyogenes* by Inducing Hydrogen Peroxide Production

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The Su strain of *Streptococcus pyogenes* (*S-coccus*)-derived anticancer preparation, OK-432, which is immobilized by heating in the presence of penicillin G, is well known to have an immunopotentiating activity through activation of natural killer cells *in vivo*. In this study, a streptococcal anticancer preparation stronger than OK-432 was prepared. Live streptococci (S-cocci) of the Su strain were induced to acquire H_2O_2-producing ability by treatment with serum under aerobic conditions. The resulting preparation no longer possessed hemolytic activity, and was not viable. The serum-treated *S-coccus* preparation activated natural killer cells as well as OK-432 did, and had stronger antitumor activity than OK-432 did. These results suggest that the serum-treated *S-coccus* preparation would be a useful tool for chemotherapy, in addition to immunotherapy, for the treatment of cancer.

Key words: *Streptococcus pyogenes* — OK-432 — Hydrogen peroxide — Antiutmor effect — Natural killer cells

There are many reports concerning the cytotoxic or cytolytic effects of streptococci on various tumor cells[1,2] *in vitro*. These effects appear to be associated with group A streptococci (*S-coccus*), and seem to be closely related to their streptolysin S-forming ability, though the actual mechanisms remain to be elucidated. It has been reported that the cytotoxic activity of *S-coccus* against tumor cells is due to cell-bound streptolysin S[3,4] and that the purified streptolysin preparation was cytotoxic to Ehrlich carcinoma cells *in vitro*.[5] The antitumor effect of the Su strain of *S-coccus* on various tumor cells has been studied. This strain is a facultative anaerobe that produces hemolysins such as streptolysin S and oxygen-labile streptolysin O. Therefore, this strain is pathogenic and toxic to the host animal. However, this toxicity can be decreased by heating at 45°C in the presence of penicillin G in BBM.[6] This led to the development of OK-432 as an anticancer preparation (its commercial name is Picibanil).[2] Although OK-432 does not directly kill tumor cells *in vitro*, it has been reported to have immunopotentiating activities, such as the activation of NK cells,[7,8] macrophage activation,[9,10] complement activation,[11] and the induction of interferons[12] and interleukin 2[13] in host animals and *in vivo* experimental systems. OK-432 has been approved in Japan for use in cancer chemotherapy as an immunopotentiator.

Abbreviations: ABTS, 2-2′-azino-di[3-ethylbenzothiazoline-(6)-sulfonic acid] ammonium salt; BBM, Bernheimer's basal medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; NK cells, natural killer cells; PBS, Dulbecco's phosphate-buffered saline; PEC, peritoneal exudate cells; *S-coccus, Streptococcus pyogenes*; S-cocci, streptococci.

Active oxygen species such as hydrogen peroxide, superoxide anion and hydroxyl radicals have cytotoxic activities which result from DNA damage, lipid peroxidation, biochemical perturbations such as cytoplasmic changes[14] and gross perturbation of the cytoskeleton and plasma membrane[15] in various types of mammalian cells and tumor cells.[16–19]

In this study, in order to make a new streptococcal anticancer preparation possessing stronger cell-killing and immunopotentiating activities than OK-432, we developed a preparation with H_2O_2-producing ability *in vitro* and then investigated its usefulness as a chemotherapeutic agent against cancer in mice.

MATERIALS AND METHODS

Materials Calbonyl iron, catalase (bovine liver) and trypan blue were purchased from Sigma (St. Louis, MO). HRP was obtained from Boehringer-Mannheim (Tokyo). ABTS was purchased from Wako Pure Chemical Industry Co., Ltd. (Osaka). Nutrient Broth, RPMI-1640, HBSS, PBS and PBS (+) containing Mg^{2+} and Ca^{2+} were obtained from Nissui Pharmaceutical (Tokyo). FCS was obtained from Gibco BRL (Gaithersburg, MD). FC-43 emulsion (oxygen gas solubility 40.3% vol %) was obtained from The Green Cross, Osaka. The FC-43 emulsion contained perfluorotributylamine (20%, w/v), Pluronic F-68 (2.56%), NaCl (0.6%), KCl (0.034%), $MgCl_2$ (0.02%), $CaCl_2$ (0.028%), $NaHCO_3$ (0.21%), glucose (0.18%) and hydroxyethylstarch (3.0%). $Na_2{}^{51}CrO_4$ (300 mCi/mgCr) was obtained from Amersham Japan (Tokyo).

Animals Female ddY, C3H/He and BALB/c strains of mice weighing 20–24g were purchased from Japan SLC

Inc. (Shizuoka) and maintained under specific pathogen-free conditions.

Organisms and culture conditions *S-coccus*, Su strain, ATCC 21060, which is stocked in our laboratory, was used. These bacteria were seeded into 0.5 ml of Nutrient Broth (meat extract, 5 g/liter; peptone 15 g/liter; NaCl 5 g/liter; K_2HPO_4, 5 g/liter; pH 7.0, Nissui) and grown at 37°C for 24 h and the precultured suspension was inoculated into 100 ml of Nutrient Broth and grown aerobically, but without shaking, for 24 h at 37°C. The bacteria were then harvested by centrifugation at 2000*g* for 20 min. They were washed twice in PBS, then used in the experiments as live S-cocci. Cell growth was monitored by measuring the absorbance at 660 nm. One ml of cell suspension with an absorbance of 0.5 at 660 nm contains 0.1 mg dry weight of bacteria. Approximately 3 to 3.5 mg dry weight of live S-cocci were usually obtained from 100 ml of culture growth.

Preparation of tumor cells Ehrlich carcinoma (ddY strain mice) cells, mouse mammary tumor (MM-2, C3H/He) cells and methylcholanthrene-induced mouse sarcoma (Meth A, BALB/c) cells were harvested from the peritoneal cavity. The harvested cells were washed with HBSS several times by centrifuging at 300*g* for 5 min. The cells were transplanted weekly in our laboratory.

Preparation of serum-treated S-cocci, heated S-cocci, OK-432, and OK-432-derived S-cocci Live S-cocci (0.3 mg) were incubated with 10% FCS in a glass test tube containing 1 ml of PBS at 37°C for 20 h under aerobic conditions. After incubation, the S-cocci were washed twice with PBS by centrifuging at 2000*g* for 15 min and then resuspended in an appropriate volume of PBS for use as the serum-treated S-cocci. Live S-cocci (0.3 mg) were incubated at 45°C for 60 min in 1 ml of PBS. After incubation, the S-cocci were washed as described above, and used for the experiments as heated S-cocci. OK-432 was prepared according to the method described by Okamoto *et al.*.[2] Briefly, live S-cocci (3 mg) were harvested from 100 ml of Nutrient Broth culture, and suspended in 30 ml of BBM,[6] which contains 25 m*M* maltose, 2.2 m*M* $MgSO_4$ and 90 m*M* KH_2PO_4 and was adjusted to pH 7.0 with NaOH, with penicillin G at a concentration of 2.7×10^4 units/ml, and this suspension was incubated at 37°C for 20 min then at 45°C for 30 min. This *S-coccus* suspension (OK-432) was lyophilized and kept at 4°C for at least 10 days. Just before use in the *in vivo* anticancer experiments, the lyophilized OK-432 preparation was resuspended in 6 ml of distilled water. OK-432-derived S-cocci were prepared by washing lyophilized OK-432 with PBS 3 times.

Aerobic and anaerobic conditions Conventional room conditions were used as the aerobic conditions. Anaerobic reaction conditions were achieved by repeated clearance and purging with pure nitrogen gas. A layer of paraffin oil was placed over the reaction mixture in glass test tubes, and they were sealed with tight rubber stoppers. The assay mixture containing 10% FCS, S-cocci, and PBS was incubated at 37°C for the time period indicated. The mixture was aspirated with a microsyringe and centrifuged at 2000*g* for 15 min. H_2O_2 content in the supernatant was determined.

Preparation of oxygenated FC-43 Pure oxygen gas was immediately bubbled through an FC-43 emulsion (20% concentration) for 5 min at room temperature, and a sample of 10% oxygenated FC-43 emulsion was used for the antitumor assay within 10 min. The partial pressure of oxygen in the sample was determined using a YSI oxygen electrode, type 5500 (YSI Co., Yellow Springs), attached to a hand-made apparatus, according to the method described previously.[20]

Determination of hydrogen peroxide Quantification of H_2O_2 was carried out according to the ABTS-peroxidase method described by Putter and Becker.[21] One hundred microliters of the supernatant of the incubation mixture was added to 900 μl of ABTS-HRP mixture (2.5 m*M* ABTS, 0.2 unit of HRP, 0.1 *M* sodium acetate buffer, pH 4.7). The reaction mixture was allowed to stand at 37°C for 20 min and the absorbance of the mixture at 420 nm was measured. The amount of H_2O_2 in the mixture was calculated from the standard curve obtained using known concentrations of H_2O_2. The determination was done in triplicate.

Assay of ^{51}Cr-release (cytolysis) A ^{51}Cr-release assay was performed using a modification of the method described by Brunner *et al.*[22] Ehrlich carcinoma or Meth A cells (1×10^6) were labeled with 25 μCi of $Na_2{}^{51}CrO_4$ in 0.5 ml of RPMI-1640 containing 10% FCS for 2 h at 37°C. The labeled cells were washed in HBSS 3 times and then used in the experiments with the *S-coccus* preparations, or with NK cells from PEC. After treatment, the labeled cell suspension was centrifuged at 1500*g* for 10 min in the case of the *S-coccus* experiment, and at 500*g* for 10 min in the NK cell activity assay, and the ^{51}Cr-radioactivity released into the supernatant was counted in a well-type gamma counter. Specific ^{51}Cr-release (cytolysis, %) was calculated according to the following formula: $[(E-S)/(M-S)]\times100$, where E is the amount of experimental ^{51}Cr-release in the experiments, S is the amount of ^{51}Cr spontaneously released from the labeled cells incubated in either RPMI-1640 alone, or with 10% FCS, and M is the maximum amount of ^{51}Cr-release from labeled target cells by freezing and thawing 3 times in water. The assay was done in triplicate.

Assay of hemolytic activity Two hundred microliters of the *S-coccus* sample supernatant was mixed with 800 μl of 3% rabbit erythrocytes suspension in PBS and incubated at 37°C for 2 h. Then, the mixture was centrifuged at 1500*g* for 10 min and the hemoglobin eluted into the

supernatant was determined by measuring its absorbance at 540 nm. Maximum hemoglobin release (complete hemolysis was achieved by suspending erythrocytes in distilled water) was denoted as 5+ on an arbitrary scale, and the extent of hemolysis in each sample was calculated by comparing its optical density with that in the case of the maximum release. The assay was done in triplicate.

Assay of the viability of S-cocci The colony-forming ability assay was used as a viability assay for the *S-coccus* preparations. *S-coccus* suspension (100 μl) containing approximately 10^3 cells in PBS was spread over the entire surface of 10 ml of hardened agar medium that contained the same Nutrient Broth used in the *S-coccus* culture, and 1.5% agar in a 10 cm diameter plate. The plate was incubated at 37°C for 24 h, and the colonies that formed on the plate were counted. The assay was done in triplicate.

PEC harvested from mice treated with S-cocci, and assay of NK cell activity *S-coccus* preparation was suspended in PBS at a concentration of 0.5 mg/ml. A BALB/c mouse was given intraperitoneal injections of 0.2 ml of *S-coccus* preparation on days 1 and 3 before harvesting. PEC were harvested from the mouse using cold RPMI-1640 containing 10% FCS and were suspended in 10 ml of the medium. The suspended cells were incubated for 45 min at 37°C in the presence of 0.5 g of carbonyl iron (4.5 to 5.2 μm average particle size). Cells containing or adhering to the iron were then removed by several passages over a strong magnet. After this treatment, viable cells were assayed by means of the trypan blue dye exclusion test, and used for the cytotoxicity assay. PEC were mixed with ^{51}Cr-labeled Meth A (2×10^5 cells) cells at a ratio of 20 : 1 in 1 ml of RPMI 1640 containing 10% FCS. After centrifugation at 150*g* for 5 min, the cell suspension was incubated at 37°C for 6 h in a 5% CO_2 and humidified air environment. Then it was centrifuged at 500*g* for 10 min and the ^{51}Cr-radioactivity released into the supernatant was counted.

Assay of antitumor effect An antitumor assay was carried out in a group of 20 mice. Tumor cells (1×10^6) were suspended in 0.2 ml of PBS and intraperitoneally injected into mice. Twenty-four hours after inoculation, 0.2 ml of the sample suspended in PBS was injected intraperitoneally, daily for 7 successive days, into the mice. The life spans of mice and the number of survivors at 60 days were determined.

Sephadex G-25 gel filtration of FCS Five milliliters of FCS was run through a Sephadex G-25 column (1.5×20 cm) which had been equilibrated with 5 m*M* NH_4HCO_3. Filtration was performed with the same buffer at a flow rate of 2 ml/h and the absorbance of the filtrate was monitored at 280 nm.

Statistical analysis Results from experiments were analyzed by using Student's *t* test.

RESULTS

Cytolytic activity of live S-cocci on tumor cells *in vitro* *In vitro* cytotoxicity of *S-coccus*, the Su strain (live S-cocci), was examined using Ehrlich carcinoma cells. Live S-cocci (0.3 mg in 1 ml) induced immediate ^{51}Cr-release from ^{51}Cr-labeled Ehrlich carcinoma cells on incubation in the presence of 10% FCS, and the ^{51}Cr-release reached a maximum of 92% after 2–3 h of incubation (Fig. 1). The ^{51}Cr-release from Ehrlich carcinoma cells induced by live S-cocci in the presence of FCS was strongly inhibited by 50 μM trypan blue, which is a specific streptolysin S inhibitor,[23] but not significantly by 1000 units of catalase in the absence of 50 μM trypan blue within 5 h (Fig. 1). These results indicate that ^{51}Cr-release from, or the cytolysis of, Ehrlich carcinoma cells induced by live S-cocci within 5 h may be due mostly to streptolysin S and slightly to H_2O_2.

Oxygen and FCS-dependent H_2O_2 production in live S-cocci Live S-cocci, which were prepared by culturing in the Nutrient Broth medium, produced H_2O_2 and released it into the medium during incubation at 37°C in the presence of FCS in PBS under aerobic conditions. The H_2O_2 produced by 0.3 mg of live S-cocci in the presence of 10% FCS under aerobic conditions increased and reached approximately 580 nmol/ml after 30 h of incubation (Fig. 2). The H_2O_2 production was inhibited

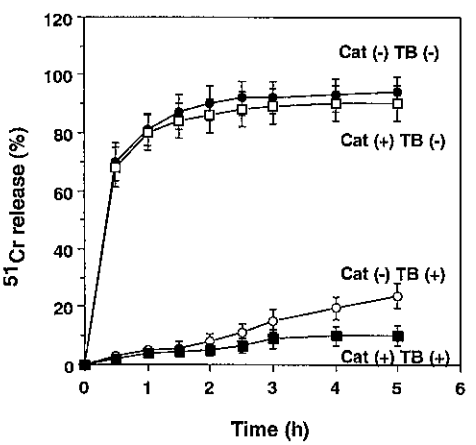

Fig. 1. Effect of trypan blue and catalase on the cytolytic activity of live S-cocci. ^{51}Cr-labeled Ehrlich carcinoma cells (1×10^6) were incubated with 0.3 mg of live S-cocci in 1 ml of PBS (+) supplemented with 10% FCS at 37°C for the times indicated, without (●), or with 1000 units of catalase (Cat) (□), with 50 μM trypan blue (TB) (○) or with 1000 units of Cat plus 50 μM TB (■), under aerobic conditions with intermittent shaking. After incubation, the ^{51}Cr-activity in the supernatant of the cell suspension was determined and is shown as specific ^{51}Cr-release. Values are the means±SD of three independent experiments.

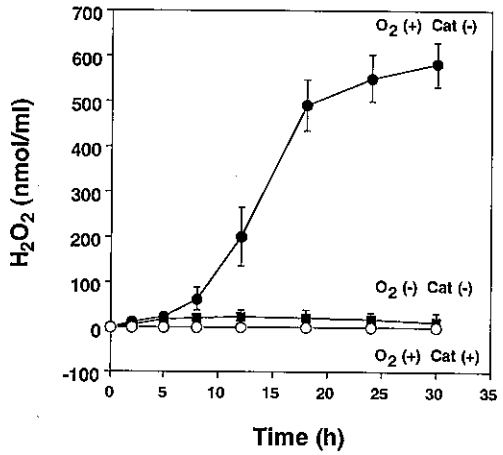

Fig. 2. Oxygen-dependent H_2O_2 production in live S-cocci in the presence of FCS. Live S-cocci (0.3 mg) were incubated with (○) or without (●, ■) 1000 units of catalase (Cat) under aerobic (○, ●) or anaerobic conditions (■) in 1 ml of PBS supplemented with 10% FCS. At the times indicated, the H_2O_2 in the supernatant of these *S-coccus* suspensions was determined. Values are the means±SD of three independent experiments.

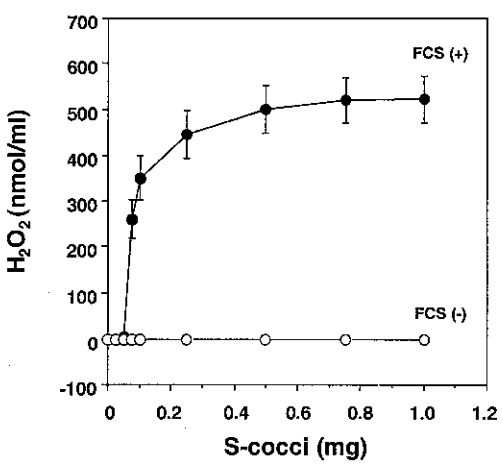

Fig. 3. H_2O_2 production in live S-cocci in the presence or absence of FCS. Live S-cocci were incubated with (●) or without (○) 10% FCS in a final volume of 1 ml with PBS at 37°C for 20 h under aerobic conditions. After incubation, H_2O_2 in the supernatant of the samples was determined. Values are the means±SD of three independent experiments.

by 1000 units of catalase and was not observed under anaerobic conditions, even though 10% FCS was present. H_2O_2 production in live S-cocci was dependent on the number of cells added (Fig. 3) and the concentration of FCS (Fig. 4). Live S-cocci (0.3 mg) produced approx-

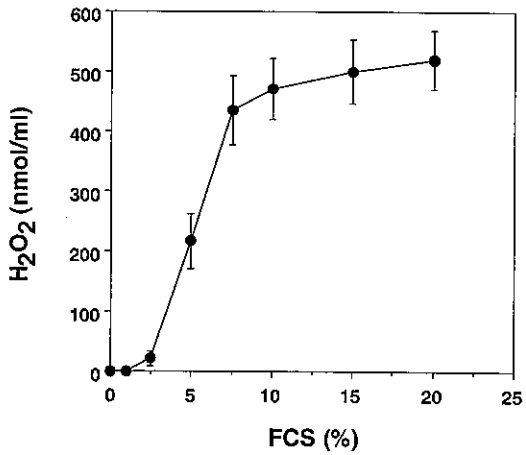

Fig. 4. FCS-dependent H_2O_2 production in live S-cocci. Live S-cocci (0.3 mg) were incubated with various concentrations of FCS in a final volume of 1 ml with PBS at 37°C for 20 h under aerobic conditions. After incubation, the H_2O_2 in the supernatant of the *S-coccus* suspensions was determined. Values are the means±SD of three independent experiments.

imately 500 μM (500 nmol/ml) H_2O_2 in the presence of 10% FCS under aerobic conditions at 37°C after 20 h of incubation (Fig. 3).

Subfractionation of FCS To isolate the factor(s) involved in H_2O_2 production in live S-cocci, FCS was fractionated by Sephadex G-25 gel filtration. Three major absorbance peaks at 280 nm were obtained by gel filtration (Fig. 5A). Fraction II contains materials having molecular sizes below 2000 daltons. The contents of fractions I, II and III were pooled and checked for the ability to induce H_2O_2 production in the *S-coccus* assay system. Only fraction II was found to be essential for the H_2O_2 production in live S-cocci. Neither fraction I nor fraction III could induce significant H_2O_2 production in live S-cocci. The effect of fraction II was not suppressed by boiling for 10 min. Sera from humans and mice could be used as substitutes for FCS (Fig. 5B).

Cytolytic and hemolytic activities and the viability of various *S-coccus* preparations Live S-cocci were obtained by culturing *S-coccus* in Nutrient Broth at 37°C for 24 h. Heated S-cocci were prepared by heating live S-cocci to 45°C for 1 h in PBS. OK-432-derived S-cocci were prepared by washing original OK-432 with PBS, which was prepared according to the method described by Okamoto *et al.*.[2] Serum-treated S-cocci were prepared by treating live S-cocci with 10% FCS for 20 h at 37°C in PBS with vigorous periodic shaking under aerobic conditions. The effects of these *S-coccus* preparations on the cytolysis of Ehrlich carcinoma cells in the presence of FCS were examined using the ^{51}Cr-releasing assay. The hemolytic activity and the viability of these *S-coccus* preparations

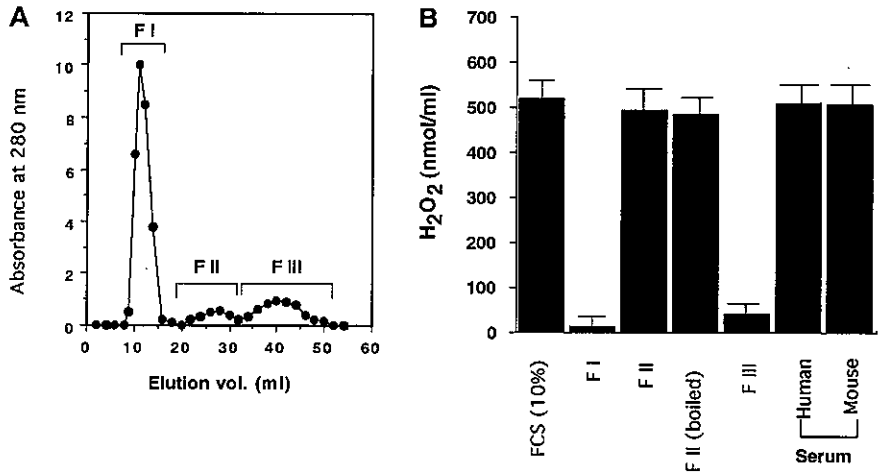

Fig. 5. Subfractionation of FCS prepared by gel filtration with Sephadex G-25, and ability of the fractions and serum to induce H_2O_2 production in live S-cocci. A, Gel filtration of FCS with Sephadex G-25 was carried out as described in the text. FI, FII and FIII indicate fraction I, II and III, respectively. B, Live S-cocci (0.3 mg) were incubated with 10% of the various eluted fractions, or 10% serum from human or mouse at 37°C for 20 h under aerobic conditions. After incubation, the H_2O_2 in the supernatant of the samples was determined. The boiled fraction II was prepared by boiling fraction II for 10 min and cooling to room temperature. Values are the means±SD of triplicate determinations from one of three independent experiments.

Table I. Cytolytic and Hemolytic Activities and the Viability of Live, Heated, OK-432-derived, and Serum-treated *S-coccus* Preparations

S-coccus preparation	Cytolysis[e] (^{51}Cr-release, %)	Hemolysis (Arbitrary unit)	Viability[f] (% living)
Live	92.6±5.5	++++	100
+Trypan blue (50 μM)	20.3±3.3	−	ND
Heated[a]	1.4±1.2	−	0
OK-432 derived[b]	8.2±3.6	+	6.5±2.3
OK-432[c]	1.8±0.6	−	0
PBS-treated[d]	36.4±5.6	++	32.6±6.4
Serum-treated	71.3±6.2	−	0
+Catalase (1000 U)	2.2±0.8	ND	ND

a) Heated S-cocci were prepared by incubating live S-cocci at 45°C for 60 min in PBS.
b) OK-432-derived *S-coccus* preparation was used after washing the original OK-432 twice in PBS.
c) Original OK-432 preparation containing penicillin G and BBM was used.
d) PBS-treated S-cocci were prepared by incubating live S-cocci without FCS in PBS at 37°C for 20 h.
e) ^{51}Cr-labeled Ehrlich carcinoma cells were incubated with 0.3 mg of *S-coccus* preparation in the presence of 10% FCS in PBS(+) for 6 h at 37°C. After incubation, ^{51}Cr released into the supernatant was determined. The results are given as mean specific ^{51}Cr-release±SD of three independent experiments.
f) The results are the mean±SD of three independent experiments.
ND, not determined.

were examined, and were expressed as arbitrary units relative to the control (live S-cocci) (Table I). Serum-treated S-cocci-induced cytolysis resulted in 71.3% ^{51}Cr-release. The ^{51}Cr-release was greatly inhibited to 2.2% by the addition of 1000 units of catalase. Neither heated S-cocci, original OK-432 nor OK-432 derived S-cocci induced large amounts of cytolysis. PBS-treated S-cocci prepared as a control for the serum-treated *S-coccus* preparation by incubation of live S-cocci in PBS without FCS caused about 36% cytolysis. Our results may indicate the partial denaturation of S-cocci by PBS treatment. The cytolytic activity of live S-cocci was decreased

to 20.3% by the addition of 50 μM trypan blue. The heated S-cocci, OK-432 derived S-cocci, and original OK-432 did not produce significant amounts of H_2O_2, even if they were incubated in the presence of 10% FCS (data not shown). The heated S-cocci, original OK-432, and serum-treated S-cocci had neither hemolytic activity nor viability. In contrast, OK-432-derived S-cocci had a low hemolytic activity and low viability.

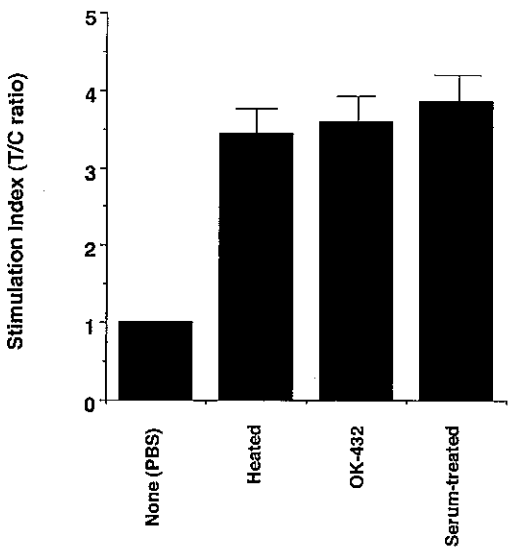

Fig. 6. Augmentation of NK cell activity by heated S-cocci, OK-432 and serum-treated S-cocci. NK cell activity was determined by means of the assay described in the text. Experiments were done in triplicate, and the results were expressed as a stimulation index (test/control) in relation to PBS treatment. The mean±SD of three independent experiments was calculated based on the mean percent of specific ^{51}Cr-release. In this experiment, the original OK-432 preparation containing penicillin G and BBM (see "Materials and Methods") was used.

Effect of *S-coccus* preparations on the augmentation of NK cell activity The immunopotentiating effects of *S-coccus* preparations on NK cell activity were examined using BALB/c mice and Meth A tumor cell systems. The NK cell activity was defined in terms of the cytolysis (^{51}Cr-release) of Meth A target cells *in vitro* (Fig. 6). There were no significant differences in NK cell activation between heated S-cocci, original OK-432, and serum-treated S-cocci. The stimulation index (T/C ratio) for serum-treated S-cocci was about 3.8.

Antitumor activity of *S-coccus* preparations on tumor cells in mice The antitumor effects of *S-coccus* preparations on various types of tumor cells in mice were assessed by antitumor activity assay, in which 0.1 mg of *S-coccus* preparation was injected intraperitoneally once a day for 7 days from one day after inoculation of the tumor cells into the peritoneal cavity (Table II). Every tumor-bearing mouse died within 2 days after the injection of live S-cocci. In all mice bearing MM-2, Ehrlich carcinoma and Meth A, the serum-treated S-cocci enhanced survival. The serum-treated S-cocci was more effective in prolonging survival than the original OK-432 or heated S-cocci. The antitumor effect of the serum-treated S-cocci was reduced by addition of 1000 units of catalase, but was enhanced by the addition of oxygenated FC-43 emulsion (10% final concentration) to the serum-treated *S-coccus* suspension, and survival time was increases more than 1.4 times in the MM-2 bearing mice, 1.3 times in the Ehrlich carcinoma cell bearing mice and 1.7 times in the Meth A-bearing mice, respectively, in comparison with OK-432. The antitumor effect of OK-432 was slightly stronger than that of heated S-cocci in mice bearing MM-2 and Ehrlich carcinoma cells. Mice inoculated for 7 days with the serum-treated *S-coccus*

Table II. Antitumor Effect of Live, Heated, OK-432 and Serum-treated *S-coccus* Preparations on Tumor-bearing Mice

S-coccus preparation	60-Day survivors (mean survival day±SD)[b]		
	MM-2	Ehrlich carcinoma	Meth A
None (PBS)	0 (20.3±1.6)	0 (19.8±2.1)	0 (15.1±1.3)
Live	0 (1.5±0.6)	0 (1.3±0.6)	0 (1.2±0.4)
Heated	12 (38.6±5.8)	8 (38.7±5.5)	8 (23.9±4.4)
OK-432[a]	13 (42.3±6.0)	9 (40.2±4.9)	8 (25.0±5.1)
Serum-treated	20	13 (49.7±6.7)*	11 (33.7±5.5)*
+Catalase (1000 U)	14 (36.3±6.7)	10 (41.0±6.0)	8 (24.6±5.4)
+Oxygenated FC-43 (337 mmHg)	20	16 (53.8±4.3)*	13 (41.7±7.1)*

a) Original OK-432 preparation containing penicillin G and BBM was used.
b) The experiment was carried out on 20 mice/group. Values represent the number of mice that were alive on the 60th day and the mean survival days±SD of the mice that died within 60 days.
* Significantly different from the mean survival days for OK-432. $P<0.05$.

preparation alone were all alive and appeared healthy on the 60th day.

DISCUSSION

Our results indicate that live *S-coccus* (Su-strain) produces H_2O_2 with the aid of unknown factor(s) contained in FCS and molecular oxygen, and that the serum-treated S-cocci have cytolytic activity against tumor cells *in vitro*, but no hemolytic effect on erythrocytes. Among substrains of *S-coccus* such as *blackmore* (which possesses the ability to produce streptolysin S), C203S (produces streptolysins S and O), and C203U (produces streptolysin O), *blackmore* produces only 10% of the H_2O_2 generated by the Su strain, and the others did not produce H_2O_2 even if they were treated with 10% FCS under aerobic conditions (data not shown). These strains are facultative anaerobes and have the same immunopotentiating activities, such as induction of interferons α and β.[24] All strains of S-cocci, except C203U, release ^{51}Cr from labeled Ehrlich carcinoma cells via the production of hemolysins, because cytolysis was inhibited by trypan blue, but not by catalase (data not shown). Our results suggest that H_2O_2 production is not related to streptolysin S production or to the hemolytic effects of the strain.

What factor(s) is needed to induce H_2O_2 production in S-cocci? The factor(s) contained in FCS or sera from various animals is heat-stable and its molecular weight is estimated to be less than 2000 daltons, as shown by Sephadex G-25 gel filtration. However, the factor has not been isolated and little is known about its chemical properties. The same factor may be present in the peritoneal cavity of mice and may be used by serum-treated S-cocci. It is unclear whether this factor activates H_2O_2 production or if it is a substrate of an intracellular enzyme(s) which produces H_2O_2, such as glucose oxidase, pyruvate oxidase or amino acid oxidase. It is known that aerotolerant anaerobes contain superoxide dismutase, but not catalase. Live S-cocci have no catalase activity, but have weak superoxide dismutase activity (data not shown). Extracts from these cells, such as *Streptococcus mutans* and *Clostridium perfringens*, contained low levels of superoxide dismutase. Some bacteria take up oxygen at rates comparable to those of aerobic organisms, and their flavoproteins use oxygen. Enzymes with oxidase activity catalyze the electron-reduction of oxygen and produce either superoxide anions, hydrogen peroxide, or water. It remains to be elucidated whether serum-treated S-cocci really have these H_2O_2-producing systems.

Since co-administration of catalase reduced the antitumor effect of the serum-treated *S-coccus* preparation on tumor-bearing mice, this *S-coccus* preparation might produce H_2O_2, especially in the peritoneal cavity of mice, using unknown substrate(s) contained in the body fluid or in the peritoneal cavity. Some biochemical perturbations in cells exposed to H_2O_2 have been reported. Hydrogen peroxide is easily reduced to the ·OH radical in the presence of Fe^{2+} via the Fenton reaction,[25] and causes biochemical responses such as lipid peroxidation,[26] irreversible inhibition of some enzymatic activities,[14, 27] DNA damage,[28] and gross perturbations of the cytoskeleton and plasma membrane.[15] However, in our case, it is unclear whether the cellular responses are caused by H_2O_2 itself, by serum-treated S-cocci, or by the ·OH radical.

Heated S-cocci did not induce ^{51}Cr-release or hemolysis, but augmented NK cell activity to a similar degree, and had a somewhat weaker antitumor effect than OK-432. The heated S-cocci or OK-432 have survival-enhancing effects on tumor-bearing mice. This implies that the cocci may not directly kill tumor cells, but may be strong immunopotentiators. NK cells are thought to be the first line of defense against cancer. NK cell activity is enhanced by OK-432.[7, 8] Serum-treated *S-coccus* preparation had a strong cytolytic activity and also augmented NK cell activity.

Oxygenated FC-43 effectively enhanced the antitumor activity of serum-treated S-cocci by providing molecular oxygen in the peritoneal cavity, where oxygen pressure may be low. FC-43 has an excellent oxygen-carrying capacity. The uptake and release of oxygen by FC-43 are completely reversible and FC-43 has been used as an oxygen carrier in the operative treatment of intestinal ischemia.[29–31] The partial pressure of oxygen in dry air is approximately 160 mmHg (0.21×760 mmHg). In mouse arterial blood, the concentration of O_2 is $\sim$0.14 m*M*, whereas in the peritoneal cavity it is probably lower than 0.06 m*M*.[32] Oxygen pressure is approximately 100 mmHg in the human pulmonary and lung aorta, but it is about 40 mmHg in the veins and it is lower than 40 mmHg in the peripheral tissue.[33] FC-43 can be used to provide oxygen to hypoxic sites *in vivo*. Enhancing effects of oxygenated FC-43 on the antitumor activity of adriamycin[34] and glucose oxidase, which produces H_2O_2 using glucose,[20] have been reported.

In conclusion, serum-treated *S-coccus* preparation possessing both immunopotentiating activity and anticancer activity may be more useful in anticancer therapy than OK-432, especially if used in conjunction with oxygenated FC-43.

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