

# Mechanism of Tumoricidal Activity of OK-432-specific L3T4<sup>+</sup> Lyt2<sup>-</sup> T-Cells

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## ABSTRACT

OK-432, a streptococcal preparation, has been used in the treatment of malignant diseases. We have found that OK-432 can act as an antigen and have established an OK-432-specific L3T4<sup>+</sup> Lyt2<sup>-</sup> T-cell line (OK2) and a clone (OK2.21) from OK-432-immunized BALB/c mice (Ia<sup>d</sup>) as antitumor effector cells. OK2 proliferated and secreted interleukin 2, but only when OK-432 was presented by Ia<sup>d</sup>-positive antigen-presenting cells. Despite its helper phenotype and function, OK2 could kill OK-432-pulsed Ia<sup>d</sup>-positive B-lymphoma cells. This killing was inhibited only by cold specific target cells (cold-target inhibition). OK-432 induced the cytotoxicity of OK2 as a specific antigen, not as a nonspecific immunostimulator. OK2 and OK2.21 also killed Ia-negative bystander target cells only in the presence of OK-432-pulsed Ia<sup>d</sup>-positive cells (bystander killing). Double-chamber experiments suggested that the bystander killing was mediated by a short-acting soluble cytolytic factor. Finally, the OK-432-specific T-cells selectively killed tumor cells, suggesting that these T-cells play an important role in the immune surveillance against malignancy.

## INTRODUCTION

Among biological response modifiers, immunostimulating agents have been utilized to augment the host's immune response against tumor cells (1). For example, OK-432, a streptococcal preparation, has been found to be effective against malignant diseases (2, 3). Several hypotheses have been proposed to explain its antitumor effects, such as augmentation of natural killer activity (4, 5) and the induction of the following activated cells: cytotoxic macrophages (6); cytotoxic polymorphonuclear cells (7); cytotoxic T-cells (8); and pantropic L3T4<sup>+</sup> killer cells (9). None of the cells, however, are specific for OK-432. Therefore, these hypotheses cannot fully explain the mechanisms either of the enhanced antitumor effect of OK-432 presensitization or of the existence of OK-432 responders and nonresponders (10).

Recent discoveries of killing function of some antigen-specific helper T-cells (11-13) raised the possibility that the helper-mediated cytotoxicity contributes to the immune surveillance against malignancy. In the previous paper (14), we demonstrated that OK-432 can induce helper T-cells that recognize its antigenic determinant(s) in the context of class II major histocompatibility complexes, and that such T-cells can act as anti-tumor effector cells both *in vitro* and *in vivo*. We established a L3T4<sup>+</sup> Lyt2<sup>-</sup> T-cell clone specific for OK-432 and restricted to I-E<sup>d</sup> from OK-432-immunized BALB/c mice and showed that the clone not only secreted IL-2 in the presence of OK-432-pulsed Ia<sup>d</sup>-bearing B-lymphoma cells but also killed them. The clone also killed Ia-negative bystander target cells, but only in the presence of OK-432-pulsed syngeneic APC.<sup>2</sup> Furthermore, the adoptive transfer of the clone together with OK-432 to syngeneic tumor-bearing BALB/c mice prolonged their sur-

vival (14). The concept that OK-432-specific T-cells serve as effector cells can explain both the existence of OK-432 responders and nonresponders and the enhanced antitumor effect of OK-432 presensitization, since it is well known that the T-cell response to a nominal antigen is genetically determined and is influenced by the previous sensitization.

The following questions, however, remain to be answered: (a) does OK-432 induce the cytotoxicity of OK-432-specific T cells as a specific antigen, but not as a nonspecific immunostimulator; (b) how do those T-cells kill target cells; and (c) what is the physiological target cell of those T-cells? The present study addresses these questions by investigating the *in vitro* cytotoxicity of the clone and its parental T-cell line.

## MATERIALS AND METHODS

**Antigens, Mice, and Tumor Cells.** OK-432, a lyophilized streptococcal preparation (Chugai Pharmaceutical Co., Tokyo, Japan), was suspended in RPMI 1640 (Gibco Laboratories, Grand Island, NY) before use. OVA was purchased from Sigma Chemical Co., St. Louis, MO. BALB/c and C57BL/10 mice were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan. BALB/c B-lymphoma A20.2J and AKR T-lymphoma BW5147 cell lines were maintained *in vitro*.

**Cell Cultures.** Cultures and assays were performed using RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 0.05 mM 2-mercaptoethanol (complete medium). Cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

**Antigen-specific T-Cells.** An OK-432-specific T-cell line (OK2) and a clone (OK2.21) were established from OK-432-immunized BALB/c mice as described previously (14). Briefly, BALB/c mice were given s.c. injections of 0.01 mg OK-432 emulsified 1:1 with complete Freund's adjuvant. Eight days later, the draining lymph nodes were removed and cultured in single cell suspensions with 0.005 mg/ml OK-432 in a 24-well plate (Costar No. 3524; Cambridge, MA) for 4 days. Responding T-cells were then purified by Ficoll density gradient centrifugation. The T-cells were put in resting culture at 4 × 10<sup>5</sup> cells/well with 4 × 10<sup>6</sup> syngeneic spleen cells that had been treated with 25 µg/ml mitomycin C for 20 min at 37°C and washed. Ten days later, 4 × 10<sup>6</sup> mitomycin C-treated syngeneic spleen cells plus 0.005 mg/ml OK-432 were added to initiate another stimulation. OK2 was obtained by repeating the round of stimulation and rest. OK2.21 was cloned from OK2 by a limiting dilution method (15). An OVA-specific T-cell line (OV1) was established from 0.5 mg OVA-immunized BALB/c mice in the same way as mentioned above, except that 0.1 mg/ml OVA was used to stimulate the line.

**Generation of Antigen-pulsed LPS Blasts and A20.2J Cells.** BALB/c spleen cells were incubated at 2 × 10<sup>6</sup>/ml with 50 µg/ml LPS (Sigma) for 48 h and pulsed with 0.05 mg/ml OK-432 for the last 16 h. A20.2J cells were incubated at 2 × 10<sup>5</sup>/ml with either 0.05 mg/ml OK-432 or 0.1 mg/ml OVA for 16 h.

**Cell Staining.** Cells were washed in phosphate-buffered saline containing 0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.5% bovine serum albumin, and 5% heat-aggregated normal rabbit serum (staining buffer) to prevent Fc fragment binding. Washed cells were allowed to react with a 1:1000 dilution of anti-Thy-1.2 (mouse IgM:MCA01; Serotec, Bicester, England), a 1:20 dilution of anti-Lyt-2.2 (mouse IgM:CL8922; Cedarlane Laboratories, Hornby, Ontario, Canada), a 1:500 dilution of anti-L3T4 (rat IgG2b:MAS110; Sera-Lab, Sussex, England) or staining buffer alone at 4°C for 30 min. The cells were then washed twice in staining buffer

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<sup>2</sup> The abbreviations used are: APC, antigen-presenting cell; OVA, ovalbumin; LPS, lipopolysaccharide; IL-2, interleukin 2.

and incubated with a 1:40 dilution of fluorescein isothiocyanate-conjugated anti-mouse IgM (ACC07F; Serotec) or a 1:20 dilution of fluorescein isothiocyanate-conjugated anti-rat IgG2b (FMAS112; Sera-Lab) at 4°C for 30 min. After washing, stained cells were analyzed by Ortho Spectrum III (Ortho Diagnostic Systems, Raritan, NJ).

**T-Cell Proliferation Assay.** Ten thousand T-cells were cultured in a flat-bottomed 96-well plate (Costar No. 3596) for 4 days with the antigens indicated and  $5 \times 10^5$  mitomycin C-treated BALB/c spleen cells. During the final 16 h,  $1 \mu\text{Ci}$  [*methyl*-<sup>3</sup>H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added. Cells were harvested on an automated harvester, and proliferation was estimated by scintillation counting of the <sup>3</sup>H cpm incorporated into DNA.

**Cytotoxic Assays.** One million target cells in 50  $\mu\text{l}$  of fetal calf serum were incubated with 100  $\mu\text{Ci}$  of sodium [<sup>51</sup>Cr]chromate solution (1.0 mCi/ml; New England Nuclear) for 1 h at 37°C and washed. The conventional <sup>51</sup>Cr release assay was carried out in a round-bottomed 96-well plate (Costar No. 3799) as described previously (13). The double-chamber <sup>51</sup>Cr release assay was carried out in a 24-well plate (Costar No. 3413), in which the inner chamber was separated from the outer chamber by a porous membrane with a pore size of 0.4  $\mu\text{m}$ . Twenty thousand <sup>51</sup>Cr-labeled target cells were placed in the inner chamber containing 0.15 ml of complete medium, and  $2 \times 10^5$  effector cells were placed in the outer chamber containing 0.7 ml of complete medium with or without antigens and APC as indicated. For spontaneous and maximum release, the target cells were incubated with the same volume of medium alone and 0.1 M HCl solution alone, respectively. The cells were incubated at 37°C for 24 h. Then 0.1 ml of each cell suspension in the inner chamber was individually transferred into a round-bottomed 96-well plate. The supernatants were harvested as in the conventional assay (13) and the specific lysis was calculated as

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100\%$$

## RESULTS

**Antigen Specificity in Proliferation.** We established an OK-432-specific T-cell line (OK2) and an OVA-specific T-cell line (OV1) from BALB/c mice immunized with the corresponding antigen. OK2 proliferated when OK-432 was presented by syngeneic spleen cells, but not when OVA was presented (Fig. 1). However, OK-432 failed to stimulate the OVA-specific T-cell line. These results indicate that OK-432 immunization has generated an OK-432-specific T-cell line in an antigen-specific manner, but not by a nonspecific stimulatory activity. Both OK2 and OV1 had a helper T-cell phenotype that was Thy-1.2<sup>+</sup>, L3T4<sup>+</sup> and Lyt-2.2<sup>-</sup> (Fig. 2), and OK2 secreted lymphokines such as IL-2 in an antigen-specific manner (14). Thus,

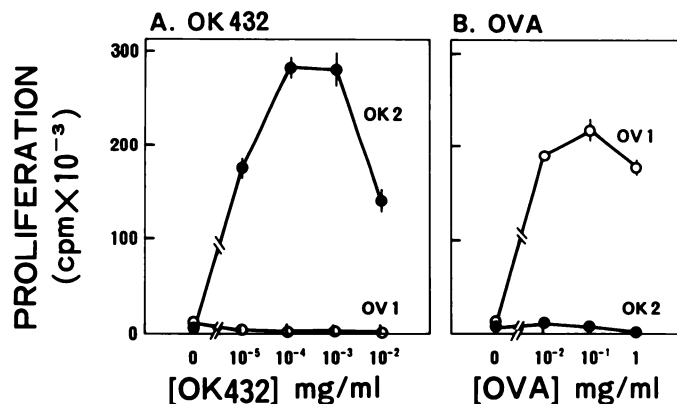


Fig. 1. Antigen-specific proliferation of OK2 and OV1. Ten thousand OK2 or OV1 cells were cultured with  $5 \times 10^5$  mitomycin C-treated BALB/c spleen cells in the presence of various concentrations of OK-432 or OVA for 4 days. During the final 16 h,  $1 \mu\text{Ci}$  [*methyl*-<sup>3</sup>H]thymidine was pulsed. Proliferation of T- or spleen cells alone was <600 cpm. Bars, SE of triplicate determinations.

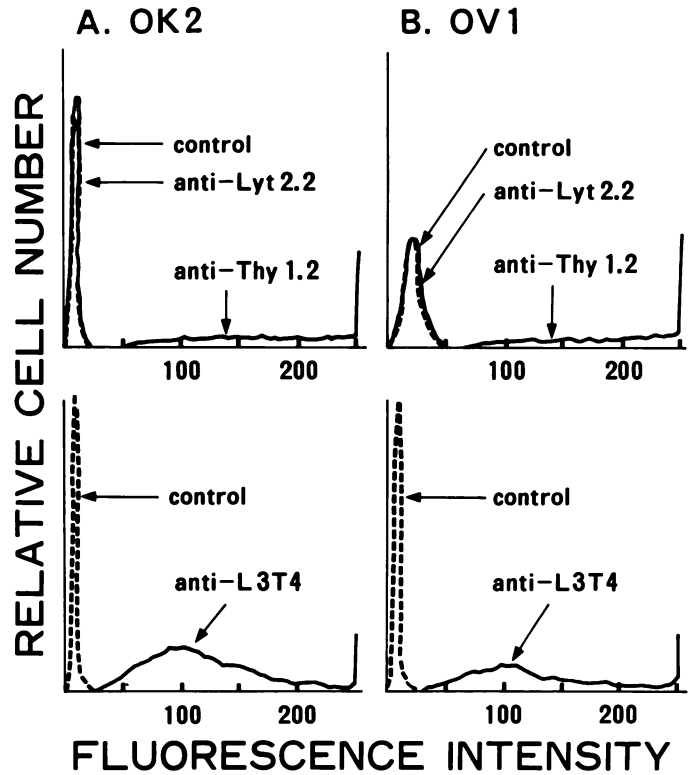


Fig. 2. Cytofluorometric profiles of OK2 and OV1. Cells were stained by an indirect method. Cells were first treated with control buffer, anti-Thy-1.2 or anti-Lyt-2.2 (top) or anti-L3T4 (bottom) followed by staining with fluorescein isothiocyanate-conjugated anti-mouse IgM (top) or fluorescein isothiocyanate-conjugated anti-rat IgG2b (bottom). Stained cells were analyzed by Ortho Spectrum III. Abscissa, relative fluorescence intensity; ordinate, cell numbers in individual fluorescence intensity channels.

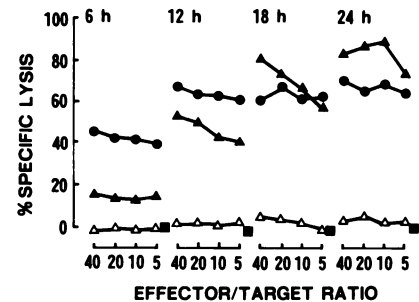


Fig. 3. Kinetics of the killing of A20.2J by OK2. The A20.2J target cells had been either pulsed with OK-432 or unpulsed before <sup>51</sup>Cr labeling. The OK-432-pulsed target was mixed with OK2 alone at the concentrations indicated (●), and the unpulsed target was mixed with either OK2 plus 0.05 mg/ml OK-432 (▲), OK2 alone (△), or 0.05 mg/ml OK-432 alone (■) in a round-bottomed 96-well plate. Cells were then incubated for the time indicated before assessing <sup>51</sup>Cr release. Spontaneous <sup>51</sup>Cr release at 6, 12, 18, and 24 h was 14–16, 17–23, 25–31, and 32–35%, respectively.

OK2 seems to be a helper T-cell line both phenotypically and functionally.

**Kinetics of Cytotoxicity.** OK2 killed Ia<sup>d</sup>-bearing B-lymphoma cells (A20.2J) in the presence of OK-432 (Fig. 3). Some OK-432-pulsed A20.2J cells were killed at 6 h, whereas 12 h and the presence of OK-432 were required for the same number of unpulsed A20.2J cells to be killed. The lag between killing of pulsed and unpulsed A20.2J cells seems to reflect the time required for OK-432 to be taken up and reexpressed by A20.2J cells, although there is no direct evidence of OK-432 in these cells. Fig. 3 also indicates that OK-432 alone or OK2 alone is not cytotoxic to A20.2J even at 24 h. Thus, the cytotoxicity of OK2 can be induced only via the recognition of OK-432 antigens expressed on the Ia<sup>d</sup>-bearing target cells.

**Antigen Specificity in Cytotoxicity.** OK2 killed unpulsed A20.2J cells only in the presence of OK-432 (Table 1). However, even in the presence of OK-432, A20.2J was not killed by the OVA-specific T-cell line. This crisscross experiment clearly demonstrates that OK-432 cannot induce the cytotoxicity of irrelevant T-cells which are not OK-432-specific. It appears that OK-432 promotes the initial interactions between effector and target cells as a specific antigen.

Antigen specificity was also demonstrated in cold-target inhibition experiments (Table 2). The killing of OK-432- or OVA-pulsed A20.2J cells by C57BL/10 anti-BALB/c MLC blasts was equally inhibited by the antigen-pulsed or unpulsed A20.2J. However, the killing of OK-432-pulsed A20.2J by OK2 was inhibited only by OK-432-pulsed A20.2J, but not by OVA-pulsed or unpulsed A20.2J. Similarly, the killing of OVA-pulsed A20.2J by OV1 was inhibited only by OVA-pulsed A20.2J. These results suggest that, in the helper-mediated killing as well as in the killing by cytotoxic T-cells, cold specific inhibitors can interfere with effector-to-target contact by binding to the same effector cells with higher affinity than nonspecific inhibitors. That is, whatever the mechanism of the OK2-mediated killing is, cell-to-cell contact is required for the killing to occur.

**Involvement of a Soluble Cytolytic Factor in Cytotoxicity.** OK2.21 was isolated by cloning OK2. OK2 and OK2.21 killed bystander target cells, but only in the presence of OK-432-pulsed Ia<sup>d</sup>-positive APC (14). Although the bystander killing favored the concept of soluble cytolytic factor involvement, the culture supernatants of OK2 (or OK2.21) plus OK-432 plus BALB/c spleen cells did not kill A20.2J or BW5147 cells, despite the positive IL-2 activity as the evidence for activation (data not shown). To further analyze the soluble factor involvement, a double-chamber 24-well plate was used to separate

Table 1 Antigen-specific killing of A20.2J by OK2 and OV1

T-cells	E:T <sup>a</sup>	Antigens		
		OK-432	OVA	None
OK2	4:1	54 ± 4 <sup>b</sup>	3 ± 1	-6 ± 2
	2:1	31 ± 2	4 ± 2	-5 ± 1
OV1	4:1	-2 ± 5	41 ± 3	-6 ± 1
	2:1	-2 ± 3	39 ± 4	-7 ± 1
None		-8 ± 1	-6 ± 3	0

<sup>a</sup> E:T, effector:target ratio.

<sup>b</sup> Percentage specific lysis ± SE of triplicate determinations. Five thousand <sup>51</sup>Cr-labeled A20.2J cells were incubated with T-cells of numbers indicated in the presence or absence of 0.05 mg/ml OK-432 or 0.1 mg/ml OVA for 24 h. Spontaneous <sup>51</sup>Cr release was 42%.

Table 2 Cold-target inhibition by antigen-pulsed or unpulsed A20.2J

Targets <sup>a</sup> (5 × 10 <sup>3</sup> )	Effectors (1 × 10 <sup>6</sup> )	Cold inhibitors <sup>a</sup> (4 × 10 <sup>5</sup> )			
		None	OK-432- A20.2J	OVA- A20.2J	Unpulsed A20.2J
OK-432- A20.2J	OK2	34 ± 2 <sup>b</sup>	3 ± 2	26 ± 1	35 ± 2
OK-432- A20.2J	MLC blasts <sup>c</sup>	25 ± 1	5 ± 1	5 ± 1	4 ± 0
OVA-A20.2J	OV1	16 ± 2	17 ± 4	2 ± 2	15 ± 2
OVA-A20.2J	MLC blasts	14 ± 1	0 ± 1	-5 ± 1	-5 ± 1

<sup>a</sup> A20.2J cells were cultured at 2 × 10<sup>5</sup> cells/ml with either 0.05 mg/ml OK-432 or 0.1 mg/ml OVA for 16 h before <sup>51</sup>Cr labeling. The same antigen-pulsed A20.2J cells were also used as cold inhibitors without <sup>51</sup>Cr labeling.

<sup>b</sup> Percentage specific lysis ± SE of triplicate determinations. Target cells were incubated with effector cells in the presence or absence of the cold inhibitors as indicated for 6 h (mixed lymphocyte culture blasts) or 24 h (OK2 and OV1) before assessing <sup>51</sup>Cr release. Spontaneous <sup>51</sup>Cr release was 15–18% and 33–36% at 6 and 24 h, respectively.

<sup>c</sup> C57BL/10 anti-BALB/c mixed lymphocyte culture blasts were generated *in vitro* as described previously (13).

Table 3 Killing of targets separated by a porous membrane from effectors

Experiment	Outer chamber <sup>a</sup>			Inner chamber <sup>a</sup> (%)
	T-cells	OK-432	APC <sup>b</sup>	<sup>51</sup> Cr-BW5147
1	OK2.21	+	SC	41 ± 17 <sup>c</sup>
	OK2.21	+	—	-14 ± 9
	OK2.21	—	SC	-13 ± 4
	—	+	SC	-8 ± 13
2	OK2.21	—	OK-LPS-SC	15 ± 1
	OK2.21	—	OK-A20.2J	16 ± 1
	OK2.21	—	—	-5 ± 0
	—	—	OK-LPS-SC	-6 ± 2
	—	—	OK-A20.2J	-5 ± 2

<sup>a</sup> BW5147 (2 × 10<sup>4</sup>) target cells were cultured for 24 h in the inner chamber, which was separated by a porous membrane from the outer chamber containing T-cells (2 × 10<sup>6</sup>), OK-432 (0.014 mg/ml), and/or APC (1 × 10<sup>6</sup>). As controls, the target cells were cocultured with T-cells, OK-432, and APC in the inner chamber, where specific lysis was 100% (experiment 1), 97% (experiment 2, OK-LPS-SC) and 34% (experiment 2, OK-A20.2J).

<sup>b</sup> Mitomycin C-treated APC used were BALB/c spleen cells (SC), BALB/c spleen LPS blasts pulsed with OK-432 (OK-LPS-SC), and OK-432-pulsed A20.2J cells (OK-A20.2J).

<sup>c</sup> Percentage specific lysis ± SE of triplicate determinations. Spontaneous <sup>51</sup>Cr release was 52 and 42% in experiments 1 and 2, respectively.

target and effector cells by a porous membrane (Table 3). The membrane did not allow the effector cells to directly contact the target cells under the microscopic observation. A substantial number of target cells were killed even when separated from OK2.21 plus OK-432 plus APC (experiment 1) or OK2.21 plus OK-432-pulsed APC (Experiment 2). These results suggest that OK2.21 secretes a soluble cytolytic factor after antigen recognition. Taken together with the finding that the supernatant lacked cytotoxicity, the putative cytolytic factor appears to be unstable.

In addition, it is unlikely that the induction of splenic macrophages to secrete cytolytic cytokines plays a major role in the bystander killing, since OK2.21 plus OK-432-pulsed A20.2J (without spleen cells) killed the targets (Experiment 2), and since OK-432 plus spleen cells failed to kill (Experiment 1). Furthermore, the activation of OK2.21 by OK-432 plus APC (Experiment 1) appears to be more potent than activation by OK-432-pulsed APC (Experiment 2), suggesting that the two activation pathways involve distinct mechanisms.

**Preferential Killing of Tumor Cells.** To investigate the physiological target cells of OK2.21, the sensitivity of OK-432-pulsed BALB/c spleen LPS blasts (normal APC) and that of OK-432-pulsed A20.2J cells (tumor APC) were compared. Although both APC types were killed by C57BL/10 anti-BALB/c mixed lymphocyte culture blasts to a similar extent (Fig. 4A), only the tumor APC were killed by OK2.21 (Fig. 4B). Furthermore, only the tumor APC could inhibit the killing of OK-432-pulsed A20.2J cells by OK2.21 (Fig. 4C), whereas both normal and tumor APC could induce the cytotoxicity of OK2.21 to the same extent (Fig. 4D). Thus, the normal APC cannot be killed by the helper T-cells or cannot inhibit the helper-mediated killing, despite the capability of binding to the helper T-cells with high affinity. Other normal APC, such as OK-432-pulsed BALB/c spleen cells and peritoneal exudate cells, were not killed by OK2.21 despite their OK2.21 stimulating capability, and C57BL/10 spleen LPS blasts were resistant to the bystander killing by OK2.21 (data not shown). These results indicate the tumor selectivity of the OK2.21-mediated killing, although the mechanism of the selectivity remains to be clarified.

## DISCUSSION

We were able to establish an OK-432-specific T-cell line (OK2) and a clone (OK2.21) from OK-432-immunized BALB/

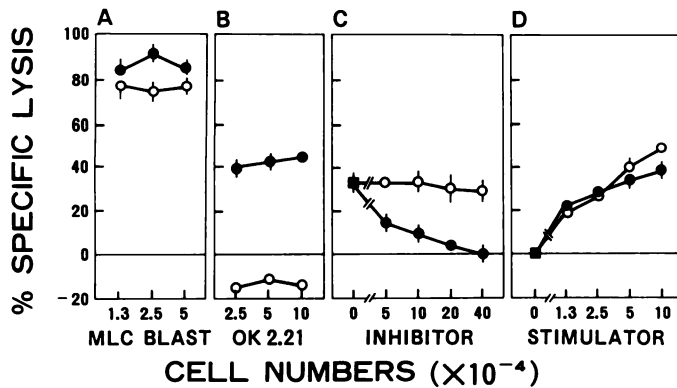


Fig. 4. Comparison of OK-432-pulsed A20.2J and OK-432-pulsed BALB/c spleen LPS blasts in sensitivity to killing by cytotoxic T-cells (A) and that by OK2.21 (B), the inhibitory activity for the OK2.21-mediated killing (C), and the stimulatory activity for the induction of the bystander killing (D). A and B, 5000 <sup>51</sup>Cr-labeled OK-432-pulsed A20.2J (●) or LPS blasts (○) were incubated with C57BL/10 anti-BALB/c mixed lymphocyte culture (MLC) blasts (A) or OK2.21 (B) of numbers indicated for 6 h. Spontaneous <sup>51</sup>Cr release was 17 and 60% in OK-432-pulsed A20.2J and LPS blasts, respectively. C, 5000 <sup>51</sup>Cr-labeled OK-432-pulsed A20.2J cells were incubated with  $2.5 \times 10^4$  OK2.21 cells for 8 h in the presence of cold OK-432-pulsed A20.2J (●) or OK-432-pulsed LPS blasts (○) at concentrations indicated, or without inhibitors (■). Spontaneous <sup>51</sup>Cr release was 20%. D, 5000 <sup>51</sup>Cr-labeled BW5147 cells were incubated with  $2.5 \times 10^4$  OK2.21 cells for 20 h in the presence of OK-432-pulsed A20.2J (●) or OK-432-pulsed LPS blasts (○) at concentrations indicated, or without stimulators (■). The stimulators alone without T-cells showed <5% specific lysis. Spontaneous <sup>51</sup>Cr release was 24%.

c mice by using the conventional method for production of exogenous antigen-specific T-cell clones. The nonspecific immunostimulating effect of OK-432 (4–9) seems to have little function in the expansion of these T-cells, since (a) OK2 proliferated only when OK-432 was presented by syngeneic APC and (b) OK-432 could not induce the proliferation of an irrelevant T-cell line. In inducing antigen-specific T-cells, however, OK-432 may be more advantageous than conventional antigens, since (a) animals may have memory T-cells specific for streptococcal antigens as a result of spontaneous infection and (b) some species may have a repertoire of such T-cells as a result of natural selection. Thus, OK-432 can evoke high T-cell response in individuals with a past history of streptococcal infection and the appropriate genetic background.

OK2 manifested not only the helper phenotype (L3T4<sup>+</sup> and Lyl2<sup>-</sup>) and function (IL-2 secretion) but also the cytotoxicity against Ia<sup>d</sup>-positive B-lymphoma cells (A20.2J) in the presence of OK-432. Crisscross experiments using an OVA-specific T-cell line indicated that it is unlikely that OK-432 kills directly the target or induces the cytotoxicity of irrelevant T-cells. On the other hand, OK-432 presumably induces the cytotoxicity of the OK-432-specific T-cells via their recognition of OK-432 antigens in the context of Ia<sup>d</sup>. The inhibition of the cytotoxicity by anti-Ia<sup>d</sup> antibodies (14) supports the idea. OK-432 is probably taken up and reexpressed by APC before the T-cells recognize it, since a time lag was observed between the killing of OK-432-pulsed A20.2J cells and that of unpulsed cells. Therefore, OK-432 acts as a nominal antigen to induce the cytotoxicity of the OK-432-specific T-cells, which can act as effector cells in the OK-432-mediated antitumor activity. In addition, other biological activities of OK-432 (4–9) also favor its antitumor activity, compared with conventional antigens such as OVA.

OK2.21 killed innocent bystander targets only during antigen-specific activation in the same manner as other helper T-cells (11–13). The bystander killing favored the hypothesis that a soluble cytolytic factor mediates the killing. However, the culture supernatant of OK2.21 plus OK-432 plus APC killed

no target cells that were otherwise sensitive to the bystander killing. Taken together with the cold-target inhibition, which indicates the requirement of cell-to-cell contact, it is likely that the soluble mediator should be unstable or effective only in the vicinity of effector cells. We observed the death of the target cells, which were separated from the effector cells by a cell-impermeable membrane. These results suggest that OK2.21 secretes a short-acting soluble cytolytic factor during antigen-specific activation.

In our previous studies (14), we demonstrated that various tumor cell lines of different major histocompatibility complex haplotypes are sensitive to the bystander killing by OK2.21. In the present study, we found that OK-432-pulsed normal BALB/c spleen cells, spleen LPS blasts, and peritoneal exudate cells are resistant to the OK2.21 cytotoxicity despite their T-cell-stimulating capability and that normal C57BL/10 spleen LPS blasts are resistant to the bystander killing by OK2.21. Therefore, OK2.21 seems to kill tumor cells selectively. The lack of a cold-inhibitory effect of normal APC suggests that the effective cold inhibitors not only bind to the effector cells with high affinity but also absorb the putative soluble mediator. The normal APC cannot inhibit the killing, presumably because they cannot absorb the mediator. To clarify the mechanism of the selectivity, the isolation of the mediator is under way.

In conclusion, our findings suggest that the OK-432-specific helper T-cells can act as antitumor effector cells by their selective tumoricidal activity in the presence of OK-432. In future OK-432 therapy, the adoptive transfer of the OK-432-specific autologous T-cells together with OK-432 can be performed without widespread killing of the host's normal cells. Furthermore, the isolation of the cytolytic mediator will influence future cancer therapy.

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