Eur J Cancer Clin Oncol, Vol. 25, No. 1, pp. 79–89, 1989.
Printed in Great Britain

0277–5379/89$3.00 + 0.00

Decrease of Transplantability by the Immunopotentiators, OK-432 and Interleukin-2: Experiments on a Human Hepatoma Cell Line in Nude Mice*

HIDETSUGU SAITO, TOSHIO MORIZANE, YASUTAKA INAGAKI, NAOKI KUMAGAI, TETSU WATANABE, TATSUYA NAKAMURA, ITSUROU SATOH, KENTAROU SAWAGUCHI, NAOTO IWABUCHI, KANJI TSUCHIMOTO and MASAHARU TSUCHIYA†

Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

Abstract—*The relationship between nonspecific cytotoxic activity of spleen cells and the resistance against the graft challenge of a human hepatoma cell line (HCC-M) was investigated in nude mice. Two administrations of an immunopotentiator, OK-432 or human interleukin-2, prior to the subcutaneous inoculation of HCC-M cells, which was performed 24 h after the last administration, significantly inhibited the tumor development in terms of rate of tumor take and tumor size. This effect was abrogated by simultaneous administration of an anti-asialo GM1 (ASGM1) antiserum. There was a significant inverse correlation between tumor volume and spleen cell cytotoxicity which was determined at the time of HCC-M cell inoculation against a YAC-1 or HCC-M target. Spleen cell cytotoxicity enhanced by these immunopotentiators could not completely be abolished by* in vitro *treatment with ASGM1 and complement. This result suggests that effector cells of the enhanced cytotoxicity consist of heterogeneous cells including both ASGM1+ natural killer cells and other nonselective cytotoxic cells. These results suggest that nonspecific cytotoxic cells play crucial roles in the resistance against tumor cell challenge and that the total level of cytotoxic activity of these cells at the time of tumor cell challenge is a key factor which determines tumor development.*

INTRODUCTION

IT IS KNOWN that the immune system is equipped with effector cells exerting nonselective cytotoxicity against a variety of tumor cells without prior sensitization. These cells include natural killer (NK) cells [1, 2], natural cytotoxic (NC) cells [3, 4] and lymphokine-activated killer (LAK) cells [5, 6]. Macrophages or monocytes are also known to have the property of destroying tumor cells in a nonselective manner when they are activated by a lymphokine [7, 8]. Although the contribution of each type of immunocyte to resistance against neoplasms *in vivo* is not fully understood, direct evidence about the role of NK cells in tumor immunology has emerged from animal experiments [9–11]. Even in human studies it has been suggested that NK cells are involved in the resistance against some types of cancer [12, 13].

In patients with liver cirrhosis (LC), the decrease of NK activity of peripheral blood mononuclear cells, as well as the impairment of general cellular immunity, have been demonstrated [14, 15]. From the present knowledge about NK cells we think that the decrease of NK activity may be one of the disadvantageous factors which make patients with LC susceptible for the development of HCC.

It is of importance to know how the activation of NK cells and other nonselective cytotoxic effector cells affects tumor development. Many biological response modifiers (BRM) are now available, including the staphylococcal preparation, OK-432, and interleukin-2 (IL-2). It has been demonstrated that they have multiple effects on the immune system. It is known that they activate NK cells [16–24], induce LAK cells [5, 6, 25–31] and activate T lymphocytes [32, 33].

Accepted 4 August 1988.
*This study was supported by a grant from the Ministry of Education, Science and Culture in Japan and a 'Grant for the Study of Hepatoma' from IBM Japan, Ltd.
†To whom correspondence and requests for reprints should be addressed at: Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

We established a human hepatocellular carcinoma (HCC) cell line, HCC-M, in 1980 [34], which is transplantable to nude mice. Nude mice are deficient in thymus and the thymus-derived immune system and as a result easily accept xenogeneic tumor cell transplantation, although NK cells and other nonselective cytotoxic cells are present [35]. Therefore, it was considered that nude mice could provide an appropriate model for studying the relationship between nonselective cytotoxicity and tumor development in a xenogeneic transplantation system.

In the present study an immunopotentiator, OK-432 or IL-2, was administered prior to the implantation of HCC-M cells; and the relationship between the nonselective cyotoxic activity of spleen cells and the tumor development was investigated to gain an insight of the role of the nonselective cytotoxicity immune system.

MATERIALS AND METHODS

Cells

HCC-M, which is a human HCC cell line established in our laboratory in 1980, from a HBsAg- and HBeAg-positive Japanese male patient [34], was used for transplantation to nude mice and as a target cell in cytotoxicity assay. Cells were grown in culture flasks (Nunc, Roskilde, Denmark) with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin (R-10 medium) (Nissui Seiyaku Co. Ltd., Tokyo, Japan) at 37°C in an atmosphere of 5% CO_2.

Medium was changed twice a week and when the cells had grown to cover the whole bottom area of the flasks they were dispersed with 0.25% trypsin in Ca^{2+}, Mg^{2+}-free 0.15 M phosphate-buffered saline (PBS) (pH 7.4) (Nakarai Chemical Inc., Kyoto, Japan), washed twice with PBS, resuspended in R-10 and transferred to other flasks.

YAC-1 cells were cultured in Dulbecco's modified essential medium (Nissui Seiyaku Co. Ltd.) supplemented with 10% FCS and the antibiotics as described above. This cell line was used as another target cell in cytotoxicity assay. The cell culture was conducted in the same way as HCC-M.

Animals

Female nude mice (BALB/c nu/nu), aged 6–8 weeks, were purchased from the Central Laboratory of Experimental Animals (Kawasaki, Kanagawa, Japan). They were grown under specific pathogen-free conditions.

Immunopotentiators and antiserum

The streptococcal preparation, OK-432, was kindly given by Chugai Pharmaceutical Co. (Tokyo, Japan). The lyophilized preparation was stored at 4°C and dissolved in PBS (0.15 M, pH 7.4) immediately before use. Purified human IL-2 (ENI IL-2) was purchased from Electronucleonics, Inc. (Colombia, MD, U.S.A.) and stored at 4°C until use. An anti-asialo GM1 (ASGM1) rabbit antiserum [36] was purchased from Wako Pure Chemical Industries (Tokyo, Japan), and stored at 4°C until use.

Treatments of animals

Immunopotentiators and ASGM1 rabbit antiserum were administered to nude mice in various combinations prior to cell implantation or afterwards according to the schedule described below. The dose of OK-432 given was 100 μg/0.2 ml per mouse and intravenously administered through a tail vein. IL-2 was given intravenously without dilution at a dose of 150 U/0.75 ml to each mouse. ASGM1 rabbit antiserum was reconstituted as the supplier recommended and diluted 1:3 with PBS and given intravenously at a dose of 0.15 ml.

Inoculation of HCC-M cells

When HCC-M cells had grown to a semiconfluent state, the culture medium was replaced with RPMI-1640 medium without FCS and cultured at 37°C for 2 days. The medium was replaced again with R-10 to facilitate the cell growth, cultured for 1 day, trypsinized as described above and dispersed cells were washed three times with PBS by centrifugation at 1000 rpm for 5 min. Cell viability was determined by Trypan blue dye exclusion. Cells were finally suspended in PBS at a concentration of 1×10^7 viable cells/ml and 0.5 ml of the cell suspension was subcutaneously injected on the back of individual nude mice by using a 1-ml plastic syringe with 21-gauge needle (Terumo, Tokyo, Japan). The injection site was closed with Alon Alpha A (Toa Gosei Chemical Industry Co., Ltd., Tokyo, Japan), which was used as a glue.

Transplantation experiment

Animals were divided into nine groups depending on the treatments they received. The experimental schedules of these groups are summarized in Table 1. Group A: 18 nude mice were used as control, to which PBS was intraperitoneally administered in a volume of 0.5 ml 1 and 3 days before the inoculation of HCC-M cells. Group B: ASGM1 rabbit antiserum was administered to eight mice 1 and 3 days before the inoculation of HCC-M cells. Group C: OK-432 was administered to eight mice 1 and 3 days before the inoculation of HCC-M cells. Group D: OK-432 and ASGM1 rabbit antiserum were simultaneously administered 1 and 3 days before the inoculation of HCC-M cells. Group E: IL-2 was administered to eight mice 1 and 3 days before the inoculation of HCC-M. Group F: IL-2 and ASGM1

Table 1. *Summary of experimental schedules*

Group of animals	No of animals	Treatment	Day(s) in relation to HCC-M cell inoculation																
			-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	18	PBS	*[1]		*	HCC-M cell inoculation					Measurement of tumor size					Measurement of tumor size					Measurement of tumor size
B	8	ASGM1	*		*																
C	8	OK-432	*		*																
D	8	OK432 + ASGM1	*		*																
E	8	IL-2	*		*																
F	8	IL-2 + ASGM1	*		*																
G	8	PBS				*			*			*			*			*			
H	8	ASGM1				*			*			*			*			*			
I	8	OK-432				*			*			*			*			*			

[1]The asterisks mean that each treatment was performed on that day.

rabbit antiserum were simultaneously administered to eight mice 1 and 3 days before the inoculation of HCC-M. In addition, three nude mice were prepared in the same way as in each group, from groups A to F, and these mice were sacrificed 24 h after the second injections and spleen cell cytotoxic activity was determined as described below. Group G: PBS was administered to eight mice at the same time as HCC-M cell inoculation and every 3 days after the inoculation. This group served as control to groups H and I. Group H: ASGM1 rabbit antiserum was administered to eight mice at the same time as HCC-M cell inoculation and every 3 days after the inoculation of HCC-M. Group I: OK-432 was administered to eight mice at the same time as HCC-M cell inoculation and every 3 days after the inoculation. In groups G, H and I the injections were given a total of 5 times. Because of the limitation of the available cell number of HCC-M, the experiment was divided into six in group A, which were performed on different occasions. In each experiment, four, three, three, three, three and two animals were used. In other groups the experiments were done on three different occasions. In each experiment, three, three and two animals were used. In each group the data were pooled and the total number of animals was as described above.

Observation of tumor development and animals

After the inoculation of HCC-M cells, the development of tumor was observed by palpating the injection site and the long axis and short axis of tumor were measured with a divider every 5 days (Table 1). The volume of the tumor was estimated according to the formula: (short axis)^2 × (long axis) × 0.4, as described by Attia and Weiss [37].

Cytotoxicity assay

Nude mice were sacrificed by neck dislocation and spleens were resected and minced with scissors into small pieces in R-10 medium. These pieces were suspended in R-10 and flushed 10 times through a 21-gauge needle attached to 10-ml plastic syringe (Terumo) and tissue debris was removed by sedimentation at 1 ***g*** for 1 min. Spleen cells were centrifuged at 1000 rpm and washed three times with R-10 and finally suspended in the same medium at concentrations of 10×10^6/ml and 5×10^6/ml.

HCC-M or YAC-1 cells in culture were trypsinized, washed twice with PBS and suspended in R-10 medium at a cell concentration of 1×10^6/ml and mixed with 100 μCi/0.1 ml $Na^{51}CrO_4$ (Tokyo Dai-ichi Radioisotope, Tokyo, Japan) and incubated at 37°C in a humid atmosphere with 5% CO_2 for 45 min. Cells were washed twice with PBS and resuspended in R-10 at a concentration of 1×10^5/ml.

Ten thousand target cells/100 μl prepared as above were dispensed into each well of a round bottomed 96-well microtiter plate (Linbro Scientific Inc., Hamden, CT, U.S.A.) and effector spleen cells prepared as described above were added in volumes of 100 μl/well in triplicate so that effector/target ratios were 100/1 and 50/1. The plates were centrifuged at 800 ***g*** for 5 min and cells were incubated at 37°C for 4 h. The plates were centrifuged at 800 ***g*** for 5 min and 100 μl of supernatants were transferred to counting vials and radioactivity was measured in a gamma counter and mean values of cpm were calculated. The maximal release was determined from the wells to which 10% saponin was added in volumes of 100 μl instead of effector

cells and spontaneous release was determined from the wells to which the culture medium was added in the same volumes instead of effector cells. The percentage cytotoxicity was calculated according to the formula:

$$\%\text{cytotoxicity} = \frac{\text{maximal release (cpm)} - \text{experimental release (cpm)}}{\text{maximal release (cpm)} - \text{spontaneous release (cpm)}} \times 100.$$

In vitro *treatment of effector cells*

Spleen cells were suspended at a cell concentration of 1×10^7/ml in ASGM1 rabbit antiserum, which was reconstituted as described above and diluted 1:20 with R-10, and incubated at 4°C for 45 min. After washing three times with R-10, cells were suspended in a guinea pig complement (Flow Laboratories, Inc., Rockville, MD, U.S.A.) diluted 1:15 with R-10 and incubated at 37°C for 45 min. Cells were washed three times with R-10, cell viability was determined by Trypan blue dye exclusion, resuspended in the same medium at appropriate concentrations and used as effector cells for cyotoxicity assay. As control, ASGM1 rabbit antiserum and the complement were replaced with PBS and cells were treated in the same way.

Determination of sequential change of cytotoxic activity after immunopotentiator administration

OK-432 or IL-2 were given once as described above to the groups of nude mice, each consisting of three animals, a half, 1, 2, 3, 4, 5 and 7 days before sacrifice. The cytotoxic activity of spleen cells was determined at the same time when they were sacrificed. As a control 0.5 ml PBS was administered to each group of three nude mice at the same time points and spleen cell cytotoxicity was determined as described above. The same experiment was repeated in the groups of nude mice, each consisting of two animals. Thus the data were pooled from each group consisting of a total of five mice.

Determination of cytotoxic activity after immunopotentiator administration

OK-432 was administered once to a group of three nude mice as described above and 12 h later they were sacrificed. The cytotoxic activity of spleen cells which were untreated or treated with ASGM1 rabbit antiserum and complement *in vitro* as described above was determined. As a control 0.5 ml PBS was administered to another group of three nude mice and the spleen cell cytotoxic activity was determined in the same way. The same experiment was repeated using two nude mice for each group and the data were pooled from five mice of each group.

IL-2 was once administered to a group of three nude mice as described above and 12 h later the cytotoxic activity of spleen cells was determined in the same way. Three mice to which 0.5 ml PBS was administered were used as a control and the spleen cell cytotoxic activity was determined in the same way. The same experiment was repeated using two nude mice for each group and the data were pooled from five mice of each group.

Statistical analysis

Group data were statistically compared by Wilcoxon's rank sum test. Correlation between two parameters was analyzed by the least square regression method. The rate of tumor take was statistically analyzed with Fisher's exact probability test.

RESULTS

Tumor take

The rate of tumor take 15 days after inoculation was 50% in group C to which OK-432 was administered and 37.5% in group E to which IL-2 was administered and 100% in group A to which PBS was administered. The difference of tumor take between group C and the control group A or between groups E and A was statistically significant with P values of <0.01 and <0.001, respectively, by Fisher's exact probability test. In other groups tumors appeared at a 100% rate (Table 2). Thus, the development of tumor was suppressed in mice to which either OK-432 or IL-2 was administered prior to tumor graft challenge, whereas the administration of OK-432 or ASGM1 rabbit antiserum after tumor challenge had no effect on tumor take,

Table 2. Rate of tumor development

Animal group [1]	No of animals	No of tumor-bearers (%) [2]	p value [3]
A	18	18 (100 %)	
B	8	8 (100 %)	NS
C	8	4 (50 %)	<0.01
D	8	8 (100 %)	NS
E	8	3 (37.5 %)	<0.001
F	8	8 (100 %)	NS
G	8	8 (100 %)	NS
H	8	8 (100 %)	NS
I	8	8 (100 %)	NS

[1]See Table 1.
[2]Determined 15 days after inoculation.
[3]Comparison between group A and each group was done according to Fisher's exact probability test. NS: not significant.

nor did the prior administration of ASGM1 rabbit antiserum alone. The suppressive effect of the prior administration of OK-432 or IL-2 on the tumor take was negated by the simultaneous administration of ASGM1 rabbit antiserum (groups D and F).

Sequential change of tumor volume

It was judged that the prior administration of ASGM1 rabbit antiserum (group B) significantly enhanced the tumor growth compared with the control group A (Table 3), since there were statistically significant differences in the tumor volumes measured 5 and 15 days after inoculation between these two groups.

The prior administration of OK-432 (group C) inhibited the tumor size (Table 3). On each day when the tumor sizes were measured there was a statistically significant difference between group C and the control group A. However, this effect of OK-432 was negated by the simultaneous administration of ASGM1 rabbit antiserum (group D) and there were no statistically significant differences of the tumor volume between groups A and D, 5 and 10 days after inoculation. However, 15 days after inoculation, the tumor volume was significantly smaller in group D than group A. Therefore, the effect of the simultaneous administration of ASGM1 rabbit antiserum was not complete in counteracting the effect of OK-432 administration, although the difference at 15 days after inoculation between these two groups was quite small.

The results similar to OK-432 were obtained with the prior administration of IL-2. On each day when the tumor sizes were measured the tumor volumes were significantly smaller in group E than the control group A. However, this effect was negated by the simultaneous administration of ASGM1 rabbit antiserum (group F) and there were no statistically significant differences of the tumor volume between groups A and F on any day when the tumor volumes were measured.

There were no statistically significant differences in the tumor volumes on any day when the tumor sizes were measured, between group H, in which ASGM1 rabbit antiserum was administered after inoculation, and the control group G, in which PBS was administered instead of ASGM1 rabbit antiserum. Similarly, there were no statistically significant differences in the tumor volume between group I, in which OK-432 was administered after inoculation, and the control group G (Table 3).

The administration of OK-432 or ASGM1 rabbit antiserum after tumor challenge had no effect on tumor volume.

Spleen cell cytotoxic activity at the time of the HCC-M cell inoculation

In the groups, each consisting of three nude mice, which were treated in the same way as in groups A to F, spleen cell cytotoxic activity was determined 24 h after the last injections, which was the same time point as the HCC-M cell inoculation in groups A to F. The cytotoxic activity was increased by the administration of OK-432 or IL-2 compared to the administration of PBS and the simultaneous administration of ASGM1 rabbit antiserum decreased the cytotoxic activity to a level which was lower than the level of the PBS administration but higher than the level of ASGM1 rabbit antiserum administration alone (Table 4). A similar

Table 3. Tumor growth

Animal group[1]	No of animals	Tumor size (mm^3)[3] mesured at indicated days after inoculation					
		5 days	p value[2]	10 days	p value	15 days	p value
A	18	48.1 ± 15.8		72.8 ± 18.4		126.2 ± 34.9	
B	8	63.1 ± 18.6	<0.05	89.2 ± 36.4	NS	162.9 ± 41.4	<0.025
C	8	12.7 ± 21.1	<0.001	5.8 ± 8.9	<0.0005	28.7 ± 35.3	<0.0005
D	8	48.1 ± 22.1	NS	84.3 ± 34.9	NS	99.3 ± 52.3	<0.005
E	8	7.5 ± 21.2	<0.001	12.6 ± 35.6	<0.005	35.5 ± 53.2	<0.001
F	8	45.9 ± 13.8	NS	67.8 ± 37.8	NS	113.4 ± 50.1	NS
G	8	46.5 ± 21.6		67.9 ± 13.4		135.1 ± 38.8	
H	8	53.5 ± 20.9	NS	84.9 ± 22.6	NS	161.5 ± 23.3	NS
I	8	33.2 ± 14.8	NS	63.9 ± 20.5	NS	149.9 ± 40.1	NS

[1]See Table 1.

[2]Statistical analysis was made between group A and each group from B to F on each day according to Wilcoxon's rank sum test. The data were also compared according to Wilcoxon's rank sum test between groups G and H or I. NS: not significant.

[3]Mean ± S.D.

Table 4. Spleen cell cytotoxic activity at the time of HCC-M cell inoculation

Group of animals [1]	%cytotoxicity against	
	YAC-1	HCC-M
A	37.9 ± 2.5 [2]	14.5 ± 1.4
B	9.4 ± 3.7	0 ± 3.5
C	65.1 ± 4.4	30.7 ± 6.7
D	21.6 ± 4.4	3.2 ± 4.1
E	63.3 ± 5.1	26.9 ± 4.3
F	16.8 ± 3.9	9.0 ± 1.2

[3]

[1]See Table 1.

[2]Mean ± S.D. of three mice.

[3]The asterisks mean that the difference is statistically significant with a P value less than 0.05 by Wilcoxon's rank sum test.

phenomenon were observed both with HCC-M and YAC-1 target cells.

The correlation between the mean value of spleen cell cytotoxic activity and the mean value of tumor volume was analyzed from the data of Tables 3 and 4. It was demonstrated that these two parameters were inversely correlated (Fig. 1). There were statistically significant correlations between the cytotoxic activity against YAC-1 cells and tumor volume determined on day 5, and that determined on day 10, and that determined on day 15. There were statistically significant correlations between the cytotoxic activity against HCC-M cells and tumor volume determined on day 5, and that determined on day 10, and that determined on day 15 (Fig. 1).

Sequential change of spleen cell cytotoxic activity after OK-432 or IL-2 administration

The level of cytotoxic activity of spleen cells determined against YAC-1 target increased even 12 h after the administration of OK-432. It reached a peak 24 h after the administration, then gradually decreased afterwards, and 4 days after the administration it decreased to a level where there was no significant difference from the level prior to OK-432 administration. Five days after administration, the level of cytotoxic activity of spleen cells increased again to the level of 2 days after the administration and on the next day it returned to the prior level (Table 5).

When IL-2 was administered, the level of cytotoxic activity of spleen cells determined against the YAC-1 target increased to reach a peak 12 h after administration, then gradually declined afterwards, and returned to the former level 2 days after the administration (Table 5).

Effect of in vitro *treatment with ASGM1 rabbit antiserum and complement on spleen cell cytotoxicity*

Normal spleen cell cytotoxic activity against YAC-1 cells and the effect of *in vitro* treatment of

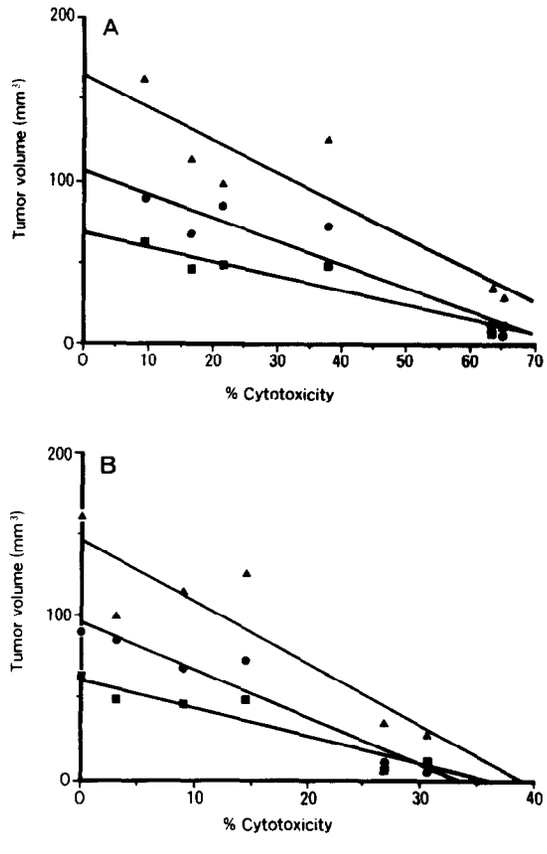

Fig. 1. Correlation between spleen cell cytotoxic activity and tumor volume. Spleen cell cytotoxic activity was determined at the same time as tumor cell challenge (see Table 4). See Table 3 for tumor volume. A: There were statistically significant correlations between the cytotoxic activity against YAC-1 cells and tumor volume determined on day 5 (■; y = 68.746 − 0.874 x, R = −0.944, P < 0.005), and that determined on day 10 (●; y = 106.636 − 1.435 x, R = −0.939, P < 0.01), and that determined on day 15 (▲; y = 165.2 − 1.986 x, R = −0.905, P < 0.025), according to the least square regression method. B: There were statistically significant correlations between the cytotoxic activity against HCC-M cells and tumor volume determined on day 5 (■; y = 61.081 − 1.674 x, R = −0.943, P < 0.005), and that determined on day 10 (●; y = 95.226 − 2.833 x, R= −0.967, P < 0.0025), and that determined on day 15 (▲; y = 147.22 − 3.764 x, R = −0.894, P < 0.025). y: tumor volume in mm³, *x: %cytotoxicity, R: correlation coefficient.*

Table 5. Sequential changes of cytotoxic activity of nude mouse spleen cells against YAC-1 cells after single administration of OK-432 or IL-2

Days after administration	OK-432		IL-2	
	% cytotoxicity	p value[1]	% cytotoxicity	p value
0	24.1 ± 7.1[2]		24.1 ± 7.1	
1/2	52.3 ± 8.2	<0.001	60.3 ± 10.3	<0.001
1	58.4 ± 18.3	<0.01	40.2 ± 13.2	<0.05
2	47.3 ± 10.4	<0.01	33.3 ± 11.5	NS
3	40.2 ± 15.5	NS	30.2 ± 9.8	NS
4	35.3 ± 12.2	NS	35.3 ± 10.6	NS
5	48.3 ± 17.6	<0.05	27.2 ± 11.4	NS
6	ND		ND	
7	30.0 ± 9.1	NS	25.3 ± 15.1	NS

[1]Cytotoxic activity was compared between day 0 and each day by Wilcoxon's rank sum test.
[2]Mean ± S.D. of five mice.

spleen cells with ASGM1 rabbit antiserum and complement is shown in Table 6. The percentage cytotoxicity increased in a dose-dependent manner by increasing the effector/target (E/T) ratio. It was decreased to negligible levels at any E/T ratio by the treatment with ASGM1 rabbit antiserum and complement. *In vitro* treatment with either ASGM1 rabbit antiserum or complement alone had no effect (data not shown). This result means that the cytotoxic effector cells are sensitive to ASGM1 rabbit antiserum which is known to recognize mouse NK cells, and that nearly all NK cells are eliminated by *in vitro* treatment.

Effect of in vitro *treatment with ASGM1 rabbit antiserum and complement on spleen cell cytotoxic activity enhanced by immunopotentiators*

When the spleen cell NK activity was increased, namely 12 h after the administration of OK-432, the spleen cells were treated *in vitro* with ASGM1 rabbit antiserum and complement and the cytotoxic activities against HCC-M and YAC-1 were determined. With this treatment the cytotoxic activities were decreased to the levels of control animals, to which PBS was given 12 h before the cytotoxicity assay. Similar results were obtained with both target cells (Table 7). However, when the spleen cells from the control animals were treated with ASGM1 rabbit antiserum and complement, the cytotoxic activities similarly determined against HCC-M or YAC-1 were further decreased to nearly negligible levels. The same results were obtained with both the target cells.

Twelve hours after the administration of IL-2, when the spleen cell cytotoxic activity was increased, the spleen cells were treated in the same way as above and the cytotoxic activities against HCC-M and YAC-1 were determined. With this treatment the cytotoxic activities were decreased to the levels of control animals, to which PBS was given 12 h before the cytotoxicity assay. Similar results were obtained with both the target cells (Table 7).

Table 6. Cytotoxic activity of spleen cells from untreated nude mice against YAC-1 and effect of in vitro *treatment with ASGM1 and complement*

Effector/target ratio	12.5	25	50	100
Untreated	9.6 ± 1.5[1]	11.4 ± 1.4	15.2 ± 2.0	23.1 ± 2.0
Treated with ASGM1 and complement	1.3 ± 0.8 *[2]	2.8 ± 0.6 *	5.3 ± 1.2 *	4.4 ± 0.4 *

[1]Mean ± S.D. of three animals.
[2]The cytotoxic activity was compared between the untreated and treated groups with Wilcoxon's rank sum test. The asterisks mean a P value less than 0.05.

Table 7. Effect of in vitro *treatment of spleen cells with ASGM1 and complement on their cytotoxic activity*

Animal treatment in vivo	Spleen cell treatment in vitro	%cytotoxicity against YAC-1		%cytotoxicity against HCC-M	
PBS	PBS	24.6 ± 5.2[1]	p<0.001[2]	3.6 ± 4.7	p<0.05
PBS	ASGM1 & complement	4.0 ± 1.3		1.2 ± 3.2	
			NS		NS
OK-432	PBS	52.6 ± 18.4	p<0.005	11.7 ± 7.3	p<0.01
OK-432	ASGM1 & complement	20.3 ± 7.6		4.6 ± 4.5	
			NS		NS
IL-2	PBS	61.0 ± 17.7	p<0.005	13.5 ± 6.0	p<0.01
IL-2	ASGM1 & complement	28.7 ± 7.5		7.2 ± 3.3	

[1]Mean ± S.D. of five mice.
[2]Statistical analysis was done according to Wilcoxon's rank sum test.

DISCUSSION

The present study demonstrates that the administration of an immunopotentiator, OK-432 or IL-2, prior to the subcutaneous xenograft challenge of a HCC cell line, inhibits tumor development in nude mice. On the other hand tumor development is significantly enhanced by the prior administration of ASGM1 rabbit antiserum, and was demonstrated to correlate with the nonselective cytotoxic activity of spleen cells at the time of cell implantation. There was a significant inverse correlation between spleen cell cytotoxic activity determined at the time of tumor cell challenge and the volume of tumor which developed afterwards. In other words, when the cytotoxic activity of spleen cells is enhanced, tumor development is suppressed and when the cytotoxic activity of spleen cells is depressed, tumor development is enhanced.

We determined the cytotoxic activity of spleen cells against two different target cells, a mouse tumor cell line, YAC-1, which is widely used as a standard target for determining mouse NK activity [38] and a human hepatoma cell line which we had established in our laboratory [34]. When OK-432 or IL-2 was administered, the cytotoxic activity of spleen cells was increased against both target cells. Both cell lines worked as target in a similar way and the result of the cytotoxicity assay was the same with these two cell lines with regard to the relative level of cytotoxic activity. Therefore, there was no apparent selectivity in the cytotoxicity determined in the present study, suggesting that the determined cytotoxic activity repesents the cytotoxicity of nonselective effector cells, i.e. NK cells, NC cells, LAK cells and macrophages, although the extent of the contribution of each cell type is not known.

The cytotoxic activity of spleen cells from untreated mice was reduced to negligible levels with the *in vitro* treatment of effector cells with ASGM1 rabbit antiserum and complement. This result indicates that the concentrations of antiserum and complement are high enough to remove all the reactive cells. This is consistent with the results reported by other investigators [39–41]. This result also means that, in the case of untreated nude mice, the majority of the effector cells of the cytotoxicity determined *in vitro* are $ASGM1^+$ cells, i.e. NK cells.

The cytotoxic activity enhanced by the administration of immunopotentiators against both target cells was also reduced with the same *in vitro* treatment but it only decreased to the same level as that in untreated mice. Therefore, it is considered that there are two different compartments in cytotoxic effector cells enhanced by IL-2 or OK-432 administration, one consisting of cells reactive with ASGM1 and the other consisting of cells non-reactive with ASGM1. There are three possibilities to be taken into consideration. One is that the treatment of effector cells with ASGM1 rabbit antiserum and complement could not sufficiently remove all NK cells beause the concentrations of these reagents were not high enough. However, this is unlikely because of the reasons mentioned above. The second possibility is that a part of NK cells might have lost the marker recognized by ASGM1 when they are activated by OK-432 or IL-2. The third possibility is that $ASGM1^-$ and other nonselective cytotoxic cells including NC cells, LAK cells and macrophages are induced or activated. It has been reported that OK-432 activates macrophages [42], as well as NK cells [16, 35, 43] and that it induces LAK cells [31], and that IL-2 activates NK cells but also induces LAK cells [5, 6, 29]. The cytotoxicity exerted by spleen cells enhanced by immunopotentiators appeared nonselective and the enhancement occurred in a very short period, which could not permit the induction of cytotoxic T lymphocytes [11, 44–47]. Therefore, we think that the cytotoxic effector cells are both NK cells and other types of

nonselective cytotoxic cells described above, when these immunopotentiators were administered.

The present study demonstrates that a certain relationship does exist between the nonselective cytotoxic activity of spleen cells determined in an *in vitro* assay and resistance against a tumor cell challenge determined *in vivo* but may not necessarily mean that effector cells for which the cytotoxicity was determined are final effector cells which destroy implanted tumor cells *in situ*. There may be a possibility that other immunocompetent cells or other effector cell mechanisms which may be activated in parallel with nonselective cytotoxic cells are directly involved in the elimination of implanted cells. However, it seems unlikely for the following reasons that these mechanisms are involved:

Nude mice are deficient in a general T cell system, though it might not be a complete deficiency [48, 49]. Therefore, it seems unlikely that cytotoxic T lymphocytes, which belong to the T cell lineage, are involved in the elimination of implanted tumor cells. Nude mice are also generally deficient in antibody production against various antigens [50]. Therefore, it is unlikely that antibody-mediated mechanisms, such as antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity, are involved in the elimination of implanted tumor cells.

The most apparent evidence which indicates that NK cells are directly involved in the elimination of implanted tumor cells is that the suppression of increased cytotoxic activity by the simultaneous *in vivo* administration of ASGM1 rabbit antiserum reversed the effect of the immunopotentiators on tumor development. The selectivity of ASGM1 for NK cells strongly suggests that the cytotoxic cells blocked *in vivo* by ASGM1 administration are NK cells [51]. Other mechanisms activated by the administration of the immunopotentiators should not be affected by ASGM1 rabbit antiserum and these mechanisms will remain intact. Actually the spleen cell cytotoxicity against YAC-1 and HCC-M could not be completely abrogated by the administration of ASGM1 rabbit antiserum. Especially, when ASGM1 rabbit antiserum was administered simultaneously with OK-432 or IL-2 the cytotoxicity enhanced by these immunopotentiators was reduced to a level lower than that of untreated normal mice but remained at considerably higher levels. The effector cells of this remaining cytotoxic activity are considered to consist mainly of $ASGM1^-$ cells, possibly NC cells and LAK cells. Macrophages may also be involved. The most important thing this result indicates is that the level of nonselective cytotoxicity determined against YAC-1 and HCC-M is correlated with the tumor development and that the difference of involved effector cells seems irrelevant to tumor development.

There should be some relationship between nonselective cytotoxic cells of the spleen and local cytotoxic cells in the site of tumor cell challenge. Activated cytotoxic cells might leave the spleen and be recruited in the site of tumor challenge, or, when spleen nonselective cytotoxic activity is enhanced, the activity of local cytotoxic cells of the same kind might be enhanced in parallel, or both phenomena might occur.

The administration of OK-432 at the time of and after the tumor challenge (group I) had no significant effect on tumor development. Even in these cases it is likely that nonselective cytotoxic activity is enhanced by the administration of the immunopotentiator. Therefore, it is suggested that nonselective cytotoxic cells activated at the time of tumor cell inoculation are effective in the inhibition of the tumor development which takes place afterwards but a short delay of the enhancement of this cytotoxic activity in relation to tumor cell challenge results in ineffectiveness on tumor development. We think that inoculated cells may proliferate to a number, which cytotoxic cells cannot handle, before cytotoxic activity is enhanced to a level high enough to eliminate them.

As the present study shows, the enhancement of spleen cell cytotoxic activity occurs very soon after the administration of the immunopotentiators but continues only for a short period. Especially, when IL-2 is administered, the level of spleen cell cytotoxic activity returns to the former level even 2 days after the administration. Therefore, it is considered that the interaction between nonselective cytotoxic cells and inoculated tumor cells, which results in the complete or partial elimination of the tumor cells, should occur during this short period. There was a statistically significant difference of tumor size even 5 days after tumor cell inoculation between the control group and the groups to which the immunopotentiators were given. This result also suggests that these immunopotentiators work soon after their administration, at least within 5 days. This also suggests that immune mechanisms which require a long period for them to become active, such as cytotoxic T lymphocytes and antibody-dependent mechanisms, may not be involved in the elimination of implanted tumor cells.

As we reported previously, NK activity of peripheral blood is significantly decreased in patients with LC [15]. From the results of the present study it could be speculated that maintaining NK activity and probably the cytotoxic activity of other cell types at a high level with the administration of an appropriate immunopotentiator could suppress the development of HCC in these patients.

Acknowledgements—We are grateful to Ms Kumi Oogiya, Ms Fumiko Nagao and Ms Itoko Shirata for their excellent technical assistance.

REFERENCES

1. Kiessling R, Klein E, Wigzell H. Natural killer cells in the mouse. I. Cytotoxic cells with specificity for mouse moloney leukemic cells. Specificity and distribution according to genotype. *Eur J Immunol* 1975, **5**, 112–116.
2. Takasugi M, Mickey MR, Terasaki PI. Reactivity of lymphocytes from normal persons on cultured tumor cells. *Cancer Res* 1973, **33**, 2898–2902.
3. Djeu JY, Lanza E, Pastore S, Hapel AJ. Selective growth of natural cytotoxic but not natural killer effector cells in interleukin-3. *Nature* 1983, **306**, 788–791.
4. Ortaldo JR, Mason LH, Mathieson BJ, Liang SM, Flick DA, Herberman RB. Mediation of mouse natural cytotoxic activity by tumour necrosis factor. *Nature* 1986, **321**, 700–702.
5. Kedar E, Ikejiri BL, Gorelik E, Herbermann RB. Natural cell-mediated cytotoxicity *in vivo* and inhibition of tumor growth *in vivo* by murine lymphoid cells cultured with T cell growth factor (TCGF). *Cancer Immunol Immother* 1982, **13**, 14–23.
6. Rosenstein M, Yron I, Kaufman Y, Rosenberg SA. Lymphokine-activated killer cells: lysis of fresh syngeneic natural killer-resistant murine tumor cells by lymphocytes cultured in interleukin 2. *Cancer Res* 1984, **44**, 1946–1953.
7. Churchill HW Jr, Piessens WF, Sulis CA, David JR. Macrophages activated as suspension cultures with lymphocyte mediators devoid of antigen become cytotoxic for tumor cells. *J Immunol* 1975, **115**, 781–786.
8. Piessens WF, Churchill HW Jr, David JR. Macrophages activated *in vitro* with lymphocyte mediators kill neoplastic but not normal cells. *J Immunol* 1975, **114**, 293–299.
9. Hanna N, Burton RC. Definitive evidence that natural killer cells inhibit experimental tumor metastasis *in vivo*. *J Immunol* 1981, **127**, 1754–1758.
10. Hanna N, Fidler I. Relationship between metastatic potential and resistance to natural killer cell-mediated cytotoxicity in three murine tumor systems. *J Natl Cancer Inst* 1983, **66**, 1183–1190.
11. Talmage JE, Meyers KM, Prieur DJ, Starkey JR. Role of NK cells in tumor growth and metastasis in beige mice. *Nature* 1980, **284**, 622–624.
12. Hersey P, Edwards A, Honeyman M, McCarthy WH. Low natural killer cell activity in familial melanoma patients and their relatives. *Br J Cancer* 1979, **40**, 113–122.
13. Hersey P, Hobbs A, Edwards A, McCarthy WH, McGovern VJ. Relationship between natural killer cell activity in histological features of lymphocyte infiltration and partial regression of the primary tumor in melanoma patients. *Cancer Res* 1982, **42**, 363–368.
14. Morizane T, Watanabe T, Tsuchimoto K, Tsuchiya M. Impaired T cell function and decreased natural killer activity in patients with liver cirrhosis and their significance in the development of hepatocellular carcinoma. *Gastroenterol Jpn* 1980, **15**, 226–232.
15. Nakamura T, Morizane T, Watanabe T *et al.* Decreased natural killer activity in patients with liver cirrhosis. *Int J Cancer* 1983, **32**, 573–575.
16. Chirigos MA, Saito T, Talmage JE. The immunomodulatory activity of picibanil (OK-432). In: Hoshino T, Uchida A, eds. *Clinical and Experimental Studies in Immunotherapy*. Tokyo, Excerpta Medica, 1984, 20–31.
17. Conlon PJ, Hefeneider SH, Henney CS, Gilis S. The effects of interleukin 2 on primary *in vivo* immune responses. In: Fefer A, Goldstein A, eds. *The Potential Role of T Cells in Cancer Therapy*. New York, Raven Press, 1982, 113–125.
18. Herberman RB, Fukui H, Talmage J, Wiltrout R, Reynolds CW, Chirigos NA. Preclinical studies with the bacterial biological response modifier (BRM), OK-432. In: Ishida N, Klein G, eds. *Host Defense Mechanism and Immunopotentiators—Proceedings of a Workshop at the 14th International Congress of Chemotherapy*. Tokyo, University of Tokyo Press, 1986, 17–21.
19. Horie Y, Hirayama C, Matsuki T, Katsube Y. A randomized clinical trial of chemotherapy and chemoimmunotherapy after transcatheter arterial embolization for patients with unresectable hepatocellular carcinoma. In: Ota K, ed. *Clinical Application of OK-432 for Control of Cancer*. Tokyo, Excerpta Medica, 1986, 63–70.
20. Ohta M, Kano T, Nishida Y, Casanova H, Tomoa Y. Effects of immunotherapy using the nonspecific immunopotentiator, OK-432, for malignant ovarian tumors. In: Hoshino T, ed. *OK-432—Proceedings of the 13th International Congress of Chemotherapy*. Tokyo, Excerpta Medica, 1984, 237–244.
21. Shirakawa S, Karitani Y, Tanaka I *et al.* Remission maintenance immunotherapy by OK-432, a streptococcal preparation, in malignant lymphoma. In: Hoshino T, ed. *OK-432—Proceedings of the 13th International Congress of Chemotherapy*. Tokyo, Excerpta Medica, 1984, 225–236.
22. Torisu M, Katano M, Kimura Y, Itoh H, Takesue M. New approach to management of malignant ascites with a streptococcal preparation, OK-432. I. Improvement of host immunity and prolongation of survival. *Surgery* 1983, **93**, 357–364.
23. Watanabe Y, Iwa T. Imunotherapy of lung cancer: a randomized clinical trial of OK-432 immunotherapy and a review of the literature on nonspecific immunotherapy. *Int J Immunother* 1986, **2**, 51–67.
24. Yamada Y, Wakita K, Mori H, Shiraki H, Noda K. Effect of local injection of OK-432 (immunomodulating agent) on cellular immunocompetence in cervical cancer. *Arch Gynecol* 1985, **237**, 283–290.

25. Chang AE, Hyatt CL, Rosenberg SA. Systemic administration of recombinant human interleukin 2 in mice. *J Biol Response Mod* 1984, **3**, 561–572.
26. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med* 1982, **155**, 1823–1844.
27. Mule JJ, Shu S, Schwarz SL, Rosenberg SA. Adoptive immunotherapy of established pulmonary metastasis with LAK cells and recombinant interleukin-2. *Science* 1984, **225**, 1487–1489.
28. Nishimura T, Hashimoto Y. Induction of nonspecific killer T cells from non-immune mouse spleen cells by culture with interleukin 2. *Jpn J Cancer (GANN)* 1984, **75**, 177–186.
29. Rosenberg SA. Adoptive immunotherapy of cancer. *Cancer Treat Rep* 1984, **68**, 233–255.
30. Rosenberg SA, Lotze MT, Muul LM *et al.* Observation of the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* 1986, **313**, 1485–1492.
31. Saito M, Ichimura O, Kataoka M *et al.* Pronounced antitumor effect of LAK-like cells induced in the peritoneal cavity of mice after intraperitoneal injection of OK-432, a killed streptococcal preparation. *Cancer Immunol Immunother* 1986, **22**, 161–168.
32. Hojo H, Hashimoto Y. Cytotoxic cells induced in tumor-bearing rats by a streptococcal preparation (OK-432). *Jpn J Cancer (GANN)* 1981, **72**, 692–699.
33. Shaw J, Caplan B, Paetkau V, Pilarski LM, Delovitch TL, McKenzie IFC. Cellular origins of costimulator (IL2) and its activity in cytotoxic T lymphocyte responses. *J Immunol* 1980, **124**, 2231–2239.
34. Watanabe T, Morizane T, Tsuchimoto K *et al.* Establishment of a cell line (HCC-M) from a human hepatocellular carcinoma. *Int J Cancer* 1983, **32**, 141–146.
35. Herberman RB. Natural cell-mediated cytotoxicity in nude mice. In: Foch J, Giovanella BC, eds. *The Nude Mouse in Experimental and Clinical Research.* New York, Academic Press, 1978, 136–166.
36. Kasai K, Iwamori M, Nagai Y, Okumura K, Tada T. A glycolipid on the surface of mouse natural killer cells. *Eur J Immunol* 1980, **10**, 175–180.
37. Attia MAM, Weiss DW. Immunology of spontaneous carcinomas in mice. V. Acquired tumor resistance and enhancement in strain A mice infected with mammary tumor virus. *Cancer Res* 1966, **26**, 1787–1800.
38. Kiessling R, Petranyi G, Klein G, Wigzell H. Genetic variation of *in vitro* cytotoxic activity and *in vivo* rejection potential of nonimmunized semisyngeneic mice against a mouse lymphoma line. *Int J Cancer* 1975, **15**, 933–940.
39. Habu S, Hayakawa K, Okumura K, Tada T. Surface markers on natural killer cells of the mouse. *Eur J Immunol* 1979, **9**, 938–942.
40. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K, Tokunaga T. *In vivo* effect of anti-asialo GM1 antibody on natural killer activity. *Nature* 1981, **291**, 334–335.
41. Shimamura K, Habu S, Fukui H, Akatsuka A, Okumura K, Tamaoki N. Morphology and function of ganglio-*N*-tetraosylceramide-positive lymphocyte mediators of natural killer activity. *J Natl Cancer Inst* 1982, **68**, 449–455.
42. Ishii Y, Yamaoka H, Toh K, Kikuchi K. Inhibition of tumor growth *in vivo* and *in vitro* by macrophages from rats treated with a streptococcal preparation, OK-432. *Jpn J Cancer (GANN)* 1976, **67**, 115–119.
43. Oshimi K, Kano S, Takaku F, Okumura K. Augmentation of mouse natural killer cell activity by a streptococcal preparation, OK-432. *J Natl Cancer Inst* 1980, **65**, 1265–1269.
44. Erard F, Corthesy P, Nabholz M *et al.* Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J Immunol* 1985, **134**, 1644–1652.
45. Klein JR, Bevan MJ. Secretion of immune interferon and generation of cytotoxic T cell activity in nude mice are dependent on interleukin 2: age-associated endogenous production of interleukin 2 in nude mice. *J Immunol* 1983, **130**, 1780–1783.
46. Lipsick JS, Kaplan NO. Interleukin 2 is mitogenic for nu/nu and nu/+ murine spleen cells. *Proc Natl Acad Sci USA* 1981, **78**, 2398–2402.
47. Stotter H, Rude E, Wagner H. T cell factor (interleukin 2) allows *in vivo* induction of T helper cells against heterologous erythrocytes in athymic (nu/nu) mice. *Eur J Immunol* 1980, **10**, 719–722.
48. Hünig T, Bevan MJ. Specificity of cytotoxic T cells from athymic mice. *J Exp Med* 1980, **152**, 688–702.
49. Ikehara S, Pahwa RN, Fernandes G, Hansen CT, Good RA. Functional T Cells in athymic nude mice. *Proc Natl Acad Sci USA* 1984, **81**, 886–888.
50. Lozzio BB, Machado EA, Lozzio CB, Mitchell J, Wust CJ. Immunotherapy of metastases of human neoplastic cells grown in immunodeficient mice. *Cancer Immunol Immunother* 1982, **12**, 135–140.
51. Okumura K, Habu S, Kasai M. The role of NK cells in resistance of *in vivo* tumors. *Adv Exp Med Biol* 1982, **155**, 773–784.