Anti-Tumour Effect of a Streptococcal Preparation (OK-432)
1. Positive Co-Relation Between the Anti-Tumour Effect and Delayed Type Hypersensitivity Reaction to OK-432 in Mice

Reiko Makidono and Keiichi Matsuura
Department of Radiology, Faculty of Medicine, Kyushu University, Fukuoka, Japan
Atsushi Makidono
Department of Experimental Radiology, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Research Code No. : 600.4

Key Words : Streptococcal preparation, Antibody Response, Cell-mediated immunity, Neoplasm, Adjuvant to Radiotherapy

溶連菌製剤（OK-432）の抗腫瘍性
I. マウスにおけるOK-432の（OK-432にたいする）遅延型過敏症反応と相関した抗腫瘍性

九州大学医学部放射線科学教室
槇殿玲子　松浦啓一
九州大学医学部放射線基礎医学教室
槇殿敦

（昭和58年8月12日受付特別掲載）
（昭和58年12月2日最終原稿受付）

溶連菌製剤（OK-432，ピシバニール）は，担癌個体の免疫機能を非特異的に賦活（調節）し，抗腫瘍性をたかめることが知られ，放射線治療にも併用されている．本報では，OK-432投与後に必然的に成立する（OK-432にたいする）免疫反応と抗腫瘍性の関係をしらべ，その作用機序の解析と最も有効な投与法を確立することを目的とした．その結果OK-432の抗腫瘍性は主として遅延型過敏症反応の成立と一致しており，遅延型過敏症反応が持続する限り，いずれの部位の固形腫瘍にたいしても抗腫瘍性が発現する可能性が示唆された．遅延型過敏症反応に少量の抗体産生が共存する状態では，腫瘍局所にOK-432を追加投与することにより抗腫瘍性は増強された．大量の抗体産生は抗腫瘍性を消失させる結果が得られた．したがってOK-432投与により抗腫瘍性を増強させるためにはOK-432にたいする遅延型過敏症反応を持続させる投与法（投与量，投与経路等）を選ぶ必要があると考えられる．

Abstract

A streptococcal preparation, OK-432 augmented the anti-tumour immunity to a spontaneous hepatoma cell line (MH134), and concurrently induced the immune responses to its own antigens in mice. The anti-tumour activity of OK-432, therefore, was analysed in connection with the immune responses to OK-432 in

mice.

Antibody production and hypersensitivity reactions of the immediate and the delayed type, to OK-432 depended on the dosage and the route of administration as well as the strain of mice. By using solid and ascitic forms of MH134, the anti-tumour effect of OK-432 was assessed in C3H/He mice in which the antibody production and the delayed type hypersensitivity reaction completely dissociated. Rejection of the solid tumour was enhanced by OK-432 pretreatment under specific conditions: OK-432 inhibited the tumour growth to a marked extent as long as generalized delayed type hypersensitivity reaction to OK-432 was maintained in mice without requiring local application. If a little opsonic antibody was produced concomitantly with the delayed type hypersensitivity reactions, tumour rejection was enhanced by the local application of OK-432. High titers of opsonic antibody to OK-432 completely abrogated the anti-tumour effect on the solid tumour. OK-432 failed to inhibit, and even promoted the proliferation of the tumour cells in ascitic form in the peritoneal cavity.

Introduction

The bacterial preparation OK-432 (Picibanil) has been in somewhat wide clinical use in Japan in the earnest hope of enhancing the anti-tumour activity of the tumour-bearing host. OK-432 is a lyophilized preparation of an attenuated strain of Streptococcus haemolyticus. Attempts to develop the preparation originated with the observation that cancer sometimes undergoes spontaneous regression in patients following an attack of erysipelas (infection with Streptococcus haemolyticus)[1)~3)]. Subsequent studies have suggested that the preparation exerts its anti-tumour effect by direct cytotoxic effect on tumour cells [4)5)], and by activating macrophages[6)~8)]. It has also been reported that infection with certain organisms produces a high resistance towards other unrelated organisms and murine tumour cells[9)~13)].

However, due to difficulties in obtaining autologous tumour cells from enough number of the patients for the study, much remains to be elucidated about the mechanism of its anti-tumour activity and its therapeutic benefit. We, therefore, tried new approach to assist the immunotherapy: This study was designed to determine whether anti-tumour activity and the immune responses to OK-432 have a special correlation, the latter being inevitably induced after the injection(s). If so, immune responses to the agent may serve as an indicator for assessing anti-tumour effect during the OK-432 treatment in man. It revealed that anti-tumour activity coincides with the delayed-type hypersensitivity reaction to OK-432. To enchance anti-tumour activity by using OK-432, therefore, such administration method that dominatly induces the delayed-type hypersensitivity reaction to the agent may be the choice.

Some characteristics of the immune responses are also discussed.

Materials and Methods

Animals. Male mice of inbred C57BL/6Ms, AKR/Ms and C3H/HeMs strains were obtained from the breeding unit of Kyushu University, Faculty of Medicine. Eight to ten week old mice were used for the experiments.

Antigens. OK-432 (Picibanil, Chugai Pharmaceutical Co. Ltd., Japan) was dissolved in saline to an appropriate concentration before use. As an animal model of OK-432 treatment of man, that being the purpose of this study, it was given to the mice basically following the same techniques and shedules used for the patients in our clinics (to be published): It was injected *intramuscularly* every other day into the right and

---

Abbreviations: DTH (delayed type hypersensitivity), HBSS (Hanks' balanced salt soultion), PBS (phosphate buffered saline), PFC (plaque forming cells), SRBC (sheep red blood cells), 2ME (2 mercaptoethanol), VBC (veronal buffered saline).

left thighs, alternately in a dose of 0.2 ml of a suspension containing 0.05 clinical unit (KE) per ml, *subcutaneously* on alternate days into 4 different sites near the axillary or inguinal region in a total dose of 0.4 ml of a suspension containing 0.025 KE per ml, or *intravenously* in a dose of 0.4 ml of the same suspension. Sheep red blood cells (SRBC) were obtained from a single animal and stored in Alsever's solution (Japan Biotest Laboratories Inc., Tokyo, Japan). Sheep RBC were washed three times in saline before use.

Tumor cells. A transplantable mouse hepatoma line, MH134 of C3H/He origin was used[14]. The tumour cell line has been maintained by serial transplant into syngeneic mice either in the solid or ascitic form.

Tumour elimination. Each of the mice implanted with tumour cells intraperitoneally received 4 ml of HBSS (containing 5 units/ml of heparin). After gentle massage, the peritoneal exudate was collected and centrifuged at 1,000 rpm for 10 minutes. The cells were stained with PAS-Giemsa and Papanicolaou stains. A mouse was considered to have rejected the tumour cells if not even a single tumour cell was present in the smears on observing 5—7 slides.

Detection of anti-OK-432 antibodies. Blood was collected from each animal by bleeding from the retro-orbital plexus. The serum was separated and then stored at −70°C until antibody titration. Immediately before titration the serum was inactivated by incubation at 56°C for 30 minutes.

(1) Detection of complement-fixing antibody

The serum was serially diluted in the wells of microtiter U plate (Model 220—24A, Kowa Kizai Co. Ltd., Japan) with veronal buffered saline (VBS) (pH 8.0). An equal volume (0.025 ml) of OK-432 suspension (8×10^{-3} KE/ml concentration) was pipetted into each well and the mixture was allowed to react at room temperature for 30 minutes. Then 0.025 ml of 1:100 diluted guinea-pig complement (dry complement, Japan Biotest Laboratories) was added to each well. The plate was incubated for 16 hours at 4°C, and to the wells 0.025 ml of a 2×10^8/ml suspension of SRBC sensitized with 5 units of rabbit anti-SRBC antibody (hemolysin) (Japan Biotest Laboratories) were added. The plate was then incubated for a further 30 min. at 37°C. The titre of complement-fixing antibody was expressed as the weakest dilution of the serum that was capable of inducing 50% hemolysis.

(2) Titration of hemagglutinating antibody

The antigen for the estimation of antibody, OK-432-SRBC was prepared by adding 1.0 ml of OK-432 suspension (0.5 KE/ml) to each milliliter of a 2×10^8/ml suspension of tannic acid treated SRBC[15]. The serial two fold dilution of the serum were prepared in the wells of the microtitration V-plate (Model 220—25A, Kowa Kizai Co. Ltd., Japan) in accordance with the microtitration technique. An equal volume (0.025 ml) of an OK-432-SRBC suspension of a 1×10^8/ml concentration was then added to each well. The plate was incubated at 4°C for subsequent reading of agglutination titer at 3 and 24 hours. For the titration of 2 Mercaptoethanol (2ME) resistant 7S antibody, the serum was subjected to similar multiple dilution with saline containing 0.01M 2ME in the wells of the microtitration plate and, after incubation at 37°C for 30 minutes, an OK-432-SRBC suspension was added to the wells. The passive hemagglutinating antibody titre was expressed as the greatest dilution of the serum that induced complete agglutination of OK-432-SRBC.

(3) Estimation of opsonic antibody titer

Opsonizing antibody was measured by the method of Cunningham *et al.* with slight modifications[16].

Amount of opsonizing antibody to OK-432 was assayed on peritoneal exudate cells collected 48 hours after the intraperitoneal infusion of 0.5 ml horse serum in each mouse. The peritoneal exudate cells were washed twice by centrifugation at 1,000 rpm for 10 minutes with medium 199 (Difco Laboratories, U.S.A.) containing 5% syngeneic mouse serum (frozen for 2 days following extraction). Then, 1.5 ml of a suspension of the cells adjusted to a concentration of 1×10^6/ml was pipetted into tubes containing a flying coverslip, and incubated at 37°C in an CO_2-incubator (95% air, 5% CO_2) for 6 hours. The tubes were gently shaken and the medium was exchanged to remove non-adherent cells. One milliliter of OK-432 suspension (1.0 KE/ml) and the same volume of test serum (containing anti-OK-432 antibody) in different concentrations prepared by

serial dilution with medium 199 (Difco, U.S.A.) supplemented with 10% normal mouse serum, were poured on the glass-adherent cells and the mixture incubated at 37°C for 1 hour. Normal mouse serum which had been frozen and thawed once served as a control. After incubation the fraction of OK-432 not phagocyted by the cells was washed out thoroughly. The cells were fixed in methanol-acetone and stained with Gram and Giemsa stains. The percentage of phagocytic cells containing ingested streptococci was estimated by microscopic examination of 1,000 cells. From 1—3% of the glass-adherent peritoneal exudate cells were phagocytic in the presence of normal mouse serum. Accordingly, the opsonic antibody titer is expressed as the highest dilution of test serum that in three separate tests induced phagocytosis in 20% or more.

Footpad assay of immediate and delayed type hypersensitivity (DTH) reactions. Each mouse was injected with 10 μl of a OK-432 suspension (0.1 KE) in phosphate buffered saline (PBS) (pH 7.2) or 20 μl of SRBC suspension in PBS (1×10^8 SRBC) in one hind foot-pad and with the volume of diluent in the other. Three and 24 hours after antigen injection, the dorso-ventral thickness of each hind foot-pad was measured in tenths of a millimeter using a dissecting microscope and a graduated eyepiece. Specific swelling was calculated by subtracting the thickness of the foot-pad injected with PBS alone from that of the foot-pad treated with antigen, OK-432 or SRBC.

Assay of plaque-forming cells (PFC). Cell suspensions were made from the spleens of mice receiving various doses of OK-432 or immunized with 1×10^8 SRBC, PFC assay was performed by the method of Cunningham and Szenberg[17].

Histological examinations. Immediately after foot-pad assay, mouse feet were fixed in 10% formol saline, decalcified, and embedded in paraffin wax. Five μm sections were cut and stained with hematoxylin and eosin.

Statistical analysis. Results are given as the mean number of PFC or tumour size (cm^3) ± standard deviation. The means of the different groups were compared by Student's t-test. Means were considered significantly different when p was <0.01.

Results

Antibody response to OK-432

In all mouse strains studied, namely C57BL/6, C3H/He and AKR mice, OK-432 administered on alternate days in a dose of 0.01 KE resulted in the production of antibody to OK-432 by any of subcutaneous, intramuscular and intravenous route. Since almost no difference was noted in this regard between the subcutaneous and intramuscular routes, the results obtained only with the subcutaneous route in 3 inbred strain mice are shown in Fig. 1. In all strains of mice, antibody production was greater after administration by the intravenous route than by the subcutaneous or intramuscular. There was a strain difference in the production of antibodies following intravenous administration of OK-432, C57BL/6 mice yielding the highest titers.

Immediate and delayed type hypersensitivity reactions to OK-432

Ninety-six animals each of C57BL/6, AKR and C3H/He strains were divided into 12 groups and injected subcutaneously, intramuscularly or intravenously with 0.01 KE of OK-432 every other day up to a total dosage of 0.1, 0.2, 0.3 or 0.4 KE. Each group of mice was then submitted to the foot-pad test to detect the immediate and the delayed type hypersensitivity reactions. All these reacted as 0.1 KE was already a sufficient amount to induce hypersensitivity reactions in all strains of mice (Figs. 2 and 3). Since no difference was noted between the subcutaneous and intramuscular routes, only the results obtained with the subcutaneous route are shown in Fig. 2. There were strain differences in both types of hypersensitivity reactions in mice injected with OK-432 by the subcutaneous or intramuscular route: The reaction was the most intense in C57BL/6 mice, followed by AKR and C3H/He mice. The 24 hr. foot-pad swelling was more intense with the increase in subcutaneous or intramuscular doses of the preparation in all strains and almost the same

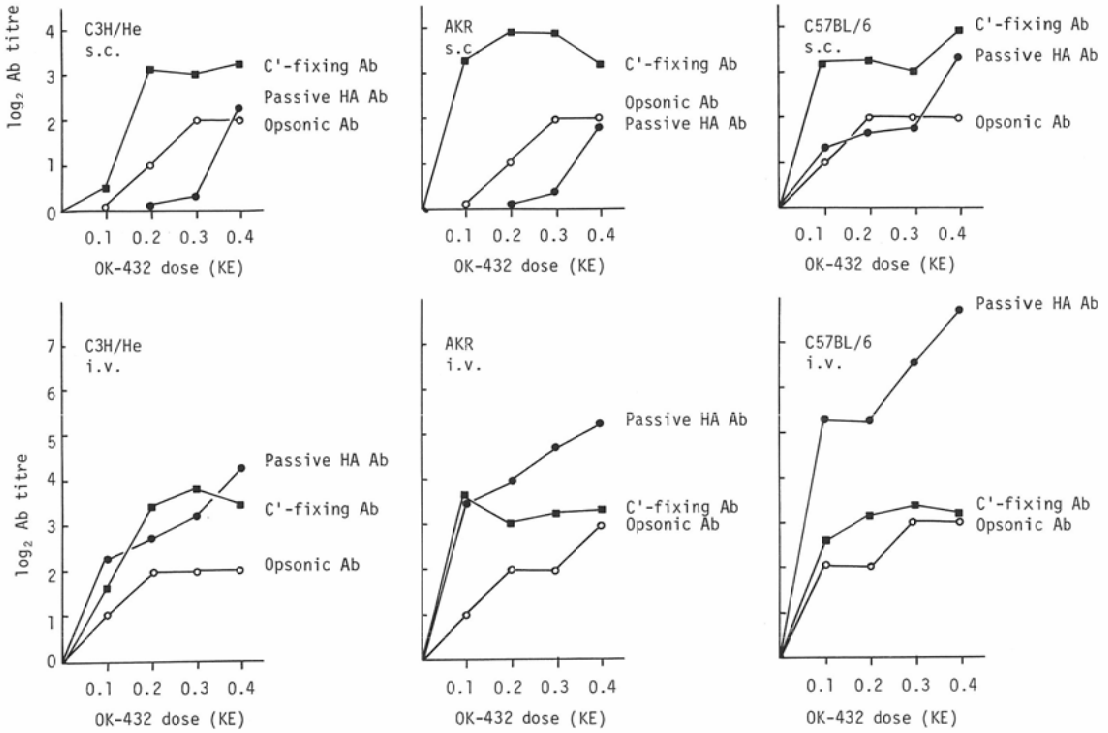

Fig. 1 Antibody response to OK-432 in mice. OK-432 was inoculated intravenously or subcutaneously every other day. When total dosage of the preparation amounted to 0.1, 0.2, 0.3 or 0.4 KE, passive hemagglutinating (●—●), complement fixing (■—■) and opsonic (○—○) antibodies were determined. Each point represents the mean of 5 ± SD.

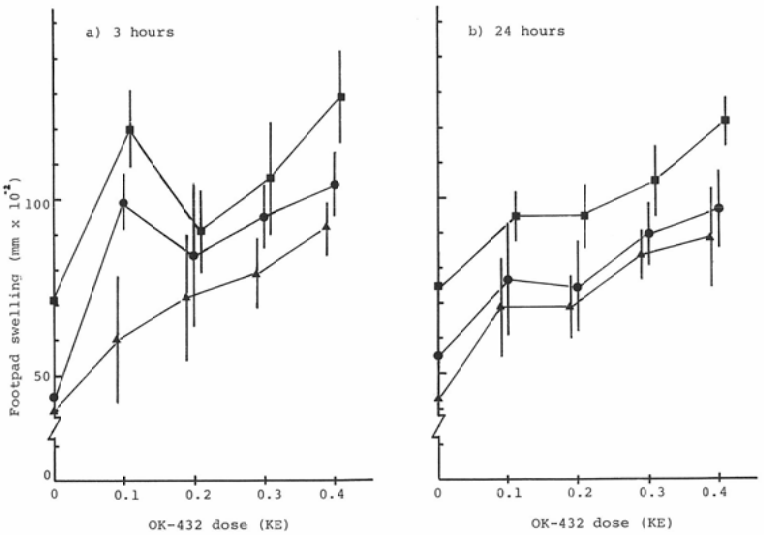

Fig. 2 Immediate and delayed-type hypersensitivity reactions to OK-432 in C3H/He (▲), AKR (●) and C57BL/6 (■) mice. Mice were administered subcutaneously with 0.01 KE of OK-432 every other day up to total dosage of 0.1, 0.2, 0.3 or 0.4 KE. Each point represents the mean of 5 ± S.D.

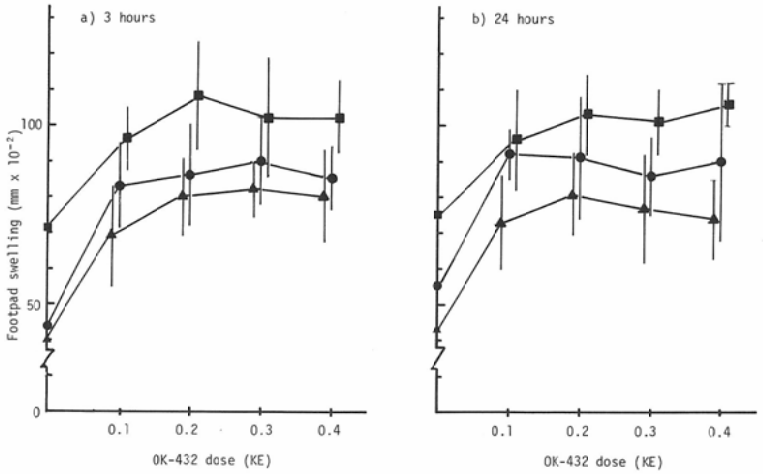

Fig. 3　Immediate and delayed-type hypersensitivity reactions to OK-432 in C3H/He (▲), AKR (●) and C57BL/6 (■) mice. Mice were administered intravenously with 0.01 KE of OK-432 every other day up to total dosage of 0.1, 0.2, 0.3 or 0.4 KE. Each point represents the mean of 5 ± SD.

degree in all strains of mice, regardless of the dose of OK-432 administered by the intravenous route (Fig. 3).

Since foot-pad thickness assay is not necessarily only a good correlate of DTH reactions[18)~22)], we also determined the type of hypersensitivity reactions induced by OK-432 in C3H/He mice by histological examinations. In non-treated mice, and mice pretreated subcutaneously with 0.1 KE of OK-432, severe interstitial oedema with a small number of infiltrating polymorphonuclear leukocytes was the outstanding histological feature of the foot-pad at 3 hrs. After 24 hrs the cellular infiltrate was predominantly mononuclear in nature (Fig. 4A). In mice pretreated subcutaneously with 0.2 or 0.3 KE of OK-432, severe interstitial oedema with an almost pure polymorphonuclear response was observed at 3 hrs and polymorphonuclear leukocytes with loose collections of eosinophils and mononuclear cells at 24 hrs (Fig. 4B). In animals receiving 0.4 KE of OK-432 by the subcutaneous route or 0.1—0.4 KE of OK-432 by the intravenous route, a moderate interstitial oedema and infiltrating polymorphonuclear leukocytes were observed at 3 and 24 hrs (Fig. 4C). These findings demonstrated that a true DTH reaction was induced in C3H/He mice by 0.1—0.3 KE subcutaneous doses of OK-432, although foot-pads remained swollen at 24 hours in all mice pretreated with 0.1—0.4 KE of OK-432.

Immune response to an unrelated antigen in OK-432 treated mice

Sixty animals each of C57/BL/6, AKR and C3H/He strain mice were divided into 4 groups and injected subcutaneously with 0.01 KE of OK-432 on alternate days. When total dosage of the preparation amounted to 0.1, 0.2, 0.3 or 0.4 KE, 5 mice of each group were injected intravenously with 1×10^6, 1×10^7, 1×10^8 of SRBC. An additional dose of SRBC (1×10^8) was injected 4 days later into the foot-pad of the mice which had received 1×10^6 or 1×10^7 SRBC to examine for the DTH reaction to SRBC. Pretreatment with OK-432 suppressed the DTH reaction to SRBC in all strain mice immunized with 1×10^7 SRBC. Mice given 0.1—0.2 KE of OK-432, however, showed enhanced DTH reaction to SRBC after intravenous immunization with 1×10^6 SRBC (Fig. 5). The number of PFC in the spleen of mice immunized with 1×10^8 SRBC were examined on the 5th day (Table 1). Mice receiving 0.1—0.2 KE of OK-432 had significantly more PFC than control mice ($p<0.01$).

Rejection of syngeneic tumour cells in OK-432 treated mice

Confirming DTH reaction and the antibody production to OK-432 in C3H/He mice (Figs. 1 and 2), we

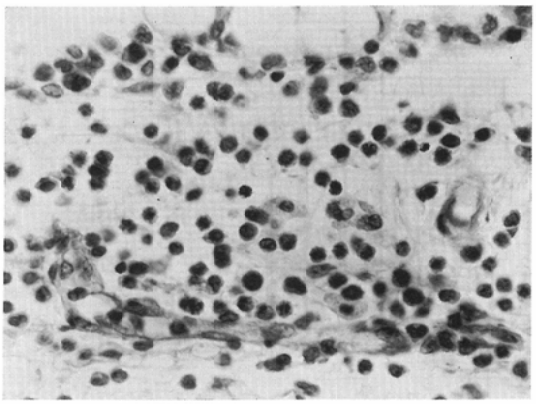

Fig. 4A Cellular response in the footpad of a C3H/He mouse treated with 0.1 KE of subcutaneous dose of OK-432. The section was prepared 24 hours after injection of OK-432 (Hematoxylin and eosin, ×940).

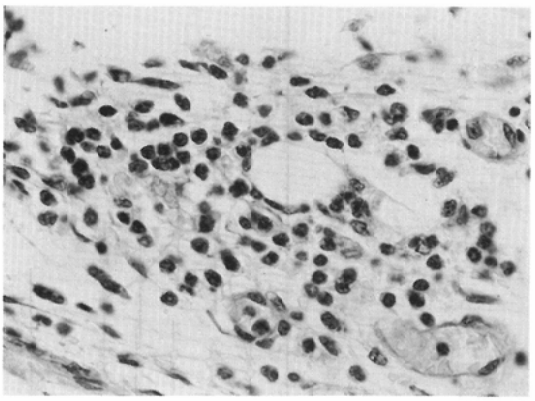

Fig. 4B Cellular response in the footpad of a C3H/He mouse treated with 0.2 KE of subcutaneous dose of OK-432 at 24 hr. (Hematoxylin and eosin, ×940).

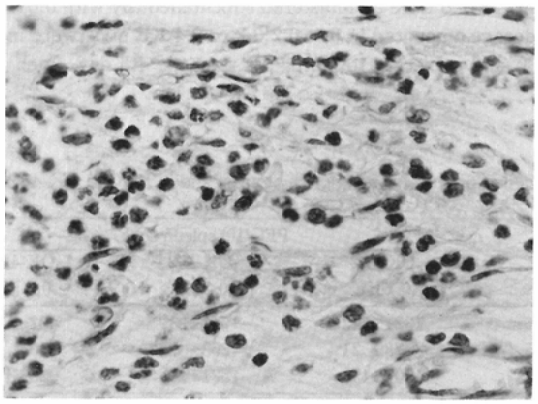

Fig. 4C Cellular response in the footpad of a C3H/He mouse treated with 0.4 KE of subcutaneous dose of OK-432 at 24 hr. (Hematoxylin and eosin, ×940).

analysed the correlation between the immune responses and the anti-tumour effect of OK-432 in this strain mice by using syngeneic MH134 tumour cells. In groups of 15 C3H/He mice receiving 0.01 KE on alternate days up to a total dosage of 0.1, 0.2 or 0.4 KE of OK-432 respectively, 1×10^5, 5×10^5 or 1×10^6 MH134 tumour cells were intraperitoneally implanted with or without 1.0 KE of OK-432. One representative of three comparable experiments is presented in Table 2. Pretreatment of mice with any dosage of OK-432 rather enhanced intraperitoneal tumour growth. An additional dose of 1.0 KE of OK-432 to the site of tumour implantation showed tumour growth enhancing effect in non-treated mice. However, virtually no effect was observed when same dose of OK-432 was administered 24 hours before implantation of the tumour cells.

A similar study was performed with 3×10^7 MH134 tumour cells implanting into the thigh muscle of non-treated as well as OK-432 treated mice (Table 3). Administration of 1.0 KE of OK-432 with the tumour implant increased rejection of the tumour at 40% in untreated mice. Mice pretreated with 0.1 KE of OK-432, having DTH reaction to OK-432, had an augmented ability to eliminate the tumour cells. In these mice an

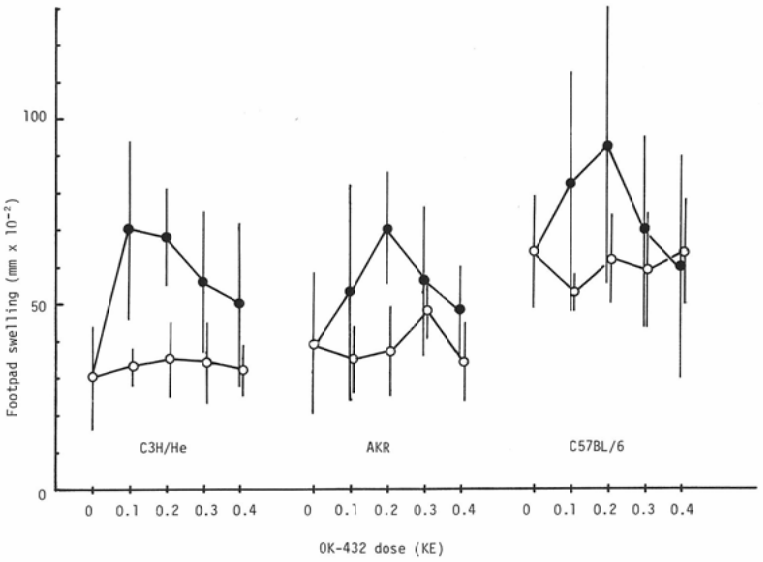

Fig. 5　Delayed-type hypersensitivity reaction to sheep RBC in OK-432 treated (●) and normal non-treated (○) mice. Mice were administered subcutaneously with 0.01 KE of OK-432 every other day. When dosage amounted to 0.1, 0.2 0.3 or 0.4 KE mice were injected intravenously with 1×10^6 SRBC and subjected to footpad test on the 4th day. Each point represents the mean of 5 ± SD.

Table 1　Number of plaque-forming cells against sheep RBC in OK-432 pretreated mice

Mice	PFC/10^6 spleen cells				
	OK-432 0 KE	OK-432 0.1 KE	OK-432 0.2 KE	OK-432 0.3 KE	OK-432 0.4 KE
C3H/He	504± 96[a)]	1321±422*	741±139	432±139	256±128
AKR	59± 25	73± 21	263± 78*	46± 16	34± 7
C57BL/6	725±152	1201±456	1521±441*	925±379	604±297

a) Mean PFC ± standard deviation. Cumulative data of 3 separate experiments (n=15).
* Significantly different by statistical analysis ($p<0.01$).

additional dose of 1.0 or 0.1 KE of OK-432 had no effect on the rejection of tumour, although tumour growth by the 7th day being markedly inhibited. In 0.2 KE pretreated mice, these having a small amount of antibody co-existing with a DTH reaction, and in 0.4 KE pretreated mice, where production of antibody to OK-432 was the predominant feature of immune response, had no ability to reject the given number of the tumour cells. Additional 1.0 or 0.1 KE of OK-432 mixed to the implanted tumour cells, however, strikingly accelerated tumour elimination in 0.2 KE pretreated mice. Only, smaller amount of OK-432 (0.1 KE) had same effect, but to a less marked extent in 0.4 KE pretreated mice.

Discussion

Growth of intramuscularly implanted tumour cells was suppressed in OK-432 pretreated mice, in which DTH reaction was predominated. In these mice additional doses of OK-432 to the site of implantation did not change the rate of elimination of tumour. However, these mice invariably had smaller tumours than those which had not received additional doses of OK-432. It is not always possible to administer OK-432 in adequate concentration directly to the site of tumour. A finding of great implication in this connection is that

Table 2 Anti-tumour effect of OK-432 on ascitic MH134 tumour cells in C3H/He mice

Dose of[a] OK-432	Treatment		Tumour-rejection on day 16
	—day 1	day 0	
0 KE	—	1×10^5 MH134 i.p	100% (20/20)[b]
	—	5×10^5 MH134 i.p	75% (15/20)
	—	1×10^6 MH134 i.p	0% (0/20)
	—	5×10^5 MH134 i.p + 1.0 KE i.p	0% (0/20)
	1.0 KE i.p	5×10^5 MH134 i.p	65% (13/20)
0.1 KE	—	5×10^5 MH134 i.p	33% (5/15)
	—	5×10^5 MH134 i.p + 1.0 KE i.p	33% (6/18)
	1.0 KE i.p	5×10^5 MH134 i.p	28% (5/18)
0.2 KE	—	5×10^5 MH134 i.p	37% (7/19)
	—	5×10^5 MH134 i.p + 1.0 KE i.p	33% (6/18)
	1.0 KE i.p	5×10^5 MH134 i.p	39% (7/18)
0.4 KE	—	5×10^5 MH134 i.p	0% (0/18)
	—	5×10^5 MH134 i.p + 1.0 KE i.p	0% (0/16)
	1.0 KE i.p	5×10^5 MH134 i.p	0% (0/20)

a) 0.01 KE of OK-432 was injected subcutaneously on alternate days up to a total dosage indicated.
b) Number of tumour-rejected mice.

Table 3 Anti-tumour effect of OK-432 on the MH134 solid tumour in C3H/He mice

Dose of[a] OK-432	Treatment		Size of tumour on day 7	Tumour-rejection on day 16
	—day 1	day 0		
0 KE	—	3×10^7 MH134 i.m	3.00±0.41[b] (10)[c]	0% (0/10)
	—	3×10^7 MH134 i.m + 1.0 KE i.m	2.27±1.04 (10)	40% (4/10)
0.1 KE	—	3×10^7 MH134 i.m	1.57±0.34 (13)*	38% (5/13)
	—	3×10^7 MH134 i.m + 1.0 KE i.m	0.62±0.24 (9)*	44% (4/ 9)
	—	3×10^7 MH134 i.m + 0.1 KE i.m	0.67±0.14 (9)*	44% (4/ 9)
0.2 KE	—	3×10^7 MH134 i.m	2.12±0.92 (11)	0% (0/11)
	—	3×10^7 MH134 i.m + 1.0 KE i.m	0.23±0.25 (10)*	80% (8/10)
	—	3×10^7 MH134 i.m + 0.1 KE i.m	0.48±0.26 (9)*	89% (8/ 9)
0.4 KE	—	3×10^7 MH134 i.m	3.25±0.36 (10)	0% (0/10)
	—	3×10^7 MH134 i.m + 1.0 KE i.m	2.98±0.91 (12)	0% (0/12)
	—	3×10^7 MH134 i.m + 0.1 KE i.m	1.18±0.51 (15)*	27% (4/15)

a) As in Table 2 footnote a)
b) Mean volume of tumours (cm^3) ± standard deviation.
c) Number of tumour-bearing mice. None of the tumours were rejected on day 7.
* significantly different from control by statistical analysis ($p<0.01$).

OK-432 requires no local application to exert its anti-tumour effect on solid tumours wherever they are located in the body as long as generalized DTH reactions are maintained (without the production of opsonic antibody). It was also shown that anti-tumour activity of OK-432, under circumstances that permit its direct application to the site of tumour, can be greatly enhanced by the presence of a minimum amount of antibody (particularly opsonic antibody) compatible with DTH reaction. It is conceivable in this case that opsonic antibody causes macrophages to ingest OK-432 in increased quantities, leading to enhanced activation of macrophages[23)24)] and/or that antigen (OK-432)-antibody complexes produced in the site of implantation lead sensitized lymphocytes and macrophages to migrate there[6)].

It has been well established by Mackaness and others that mice pretreated with various bacterial

preparations have an increased resistance to tumours and the role of the macrophage as an effector cell in non-specific cell-mediated immunity to tumours has been emphasized by a great number of investigators[5)25)~31)].

The hemolytic streptococcal organisms (Group A, type 3) from which OK-432 is prepared, possess type specific protein M and non-type-specific antigens on their surfaces[23)24)32)33)]. Of these, type-specific M protein on the surface of the organism renders it resistant to the phagocytic defense of the non-immune host. Phagocytic cells such as neutrophilic leukocytes and macrophages can efficiently digest the organism only in the presence of antibody to protein M (opsonic antibody) or antibody together with complement[23)24)33)]. A moderate titer of opsonic antibody was produced in all animal strains examined by any route of administration and was found to have both beneficial and deleterious effects on the rejection of tumour cells by the tumour-bearing host.

Among the other kinds of antibody produced at different intervals following administration of OK-432, some complement-fixing antibody is known to be related to immediate type hypersensitivity reactions[34)]. In this study immediate type hypersensitivity reaction approximately coincided with the appearance of this type of antibody.

The interrelationship between the humoral and cell-mediated immune responses to a given antigen remains largely obscure. A negative correlation often exists between the production of antibody and DTH reaction to the same antigen[12)34)~36)], and passive transfer of antibody resulted in reduced DTH reaction[37)]. There was also an inverse relationship between humoral and cell-mediated immune response to OK-432. Repeated doses of OK-432 induced heightened levels of antibody but reduced levels of DTH. When OK-432 was given intermittently at a certain intervals, antibody production was also promoted while DTH reaction became less persistent (data not shown). As to the relationship between the route of administration of OK-432 and the type of resultant immune response, DTH reaction was more readily induced with OK-432 given intramuscularly or subcutaneously than intravenously. Our experience with OK-432 in man has indicated that, in particular when it was administered intradermally, DTH reaction persisted for a long time, taking place readily and without association with antibody production, which was extremely limited (Makidono *et al.*, Ms. in preparation).

Of the animal strains used C57BL/6 exhibited the most intense immune response to both humoral and the cell-mediated types, the other 2 strains, i.e., AKR and C3H/He, being compatible in this respect. There is some evidence for the existence of genetic control in the immune response to infectious organisms[38)39)], and the same might apply to the immune response to hemolytic streptococci or OK-432, although no definitive data in support of this presumption has yet been obtained.

Enhanced immune response to SRBC, both humoral and cellular, coincided with the development of DTH reactions to OK-432. This finding suggests that OK-432 induced anti-tumour activity is immunologically non-specific and probably mediated as a consequence, either by lymphokines (mediators of cellular immunity) or by activated macrophages. Because of the fact that many tumours are susceptible to the growth inhibitory effects of macrophages, the latter possible mechanism, macrophage-mediated anti-tumour activity of OK-432, should be further analysed by using same tumour cells (MH134) as well as tumours of different origin. A study of this aspect is in progress.

Acknowledgement

We thank Mrs. Tomiko Terashima and Miss Yūko Nakayama for their secretarial assistance.

References

1) Busch, W.: Verhandlungen ärztlicher Gesellschaften. Nieder-Rheinische Gesellschaft für Natur und Heilkunde in Bonn. Aus der Sitzung der Medizinischen Section von 13. Berlin. Klin. Wochs. 5:137, 1868

2) Fehleisen: Ueber die Züchtung der Erysipelkokken auf Künstlichem Nährboden und ihre Uebertragbarkeit auf den Menschen. Deut. Med. Wochs. 8: 553, 1882
3) Coley, W.B.: Contribution to the knowledge of Sarcoma. Ann. Surg. 14: 199, 1891
4) Ohta, T.: Experimental anticancer studies. Part VI. Experiments in the influence on living A group hemolytic streptococci and several other species of microorganisms on the invasion power of Ehrlich carcinoma cells to mice. Jap. J. Exp. Med. 27: 107—116, 1957
5) Old, L.J., Clarke, D.A. and Benacerraf, B.: Effect of Bacillus Calmette-Guerin Infection on Transplanted Tumours in the Mouse. Nature 184: 291—292, 1959
6) North, R.J. and Spitalny, G.: Inflammatory lymphocyte in cell-mediated antibacterial immunity: factors governing the accumulation of mediator T cell in peritoneal exudates. Infect. Immun. 10: 489—498, 1974
7) Kimura, I., Onoshi, T., Yasuhara, S., Sugiyama, M., Urabe, Y., Hujii, M. and Machida, K.: Immunological studies on the Streptococcal agent against tumours (Report 1). Clin. Immunol. 7: 1275—1278, 1975
8) Ishii, Y., Yamaoka, H., Toh, K. and Kikuchi, K.: Inhibition of Tumor Growth in vivo and in vitro by Macrophages from Rats Treated with a Streptococcal Preparation, OK-432. Gann 67: 115—119, 1976
9) Mackaness, G.B.: The immunological basis of acquired cellular resistance. J. Exp. Med. 120: 105—120, 1964
10) Hibbs, J.B.Jr., Lambert, L.H.Jr. and Remingston, J.S.: Resistance to murine tumour conffered by chronic infection with intracellular protozoa, Toxoplasma gondii and Besnoitia jellisoni. J. Infect. Dis. 124: 587—592, 1971
11) Allen, E.G. and Mudd, S.: Protection of mice against vaccinia virus by bacterial infection and sustained stimulation with specific bacterial antigens. Infect. Immun. 7: 62—67, 1973
12) Parish, C.R.: Immune response to chemically modified flagellin. IV. Further studies on the relationship between humoral and cell mediated immunity. Cell. Immunol. 6: 66—79, 1973
13) Natsu-Ume Sakai, S., Ryoyama, K., Koshimura, S. and Migita, S.: Studies on the properties of a streptococcal preparation OK-432 (NSC-B116209) as an immunopotentiator. I. Activation of serum complement components and peritoneal exudate cells by group A streptococcus. Jap. J. Exp. Med. 46: 123—133, 1976
14) Sato, H., Kawashima, Y., Kanno, K., Munakata, H. and Saito, T.: Studies to the transplantability of the mouse ascites hepatomas. Fukushima J. Med. Sci. 5: 155—173, 1958
15) Kabat, E.A. and Mayer, M.M.: In experimental Immunochemistry. pp. 120 Charles C. Thomas, Springfield, Illinois, 1971
16) Cunningham, A.J. and Beachey, E.H.: Immunochemical properties of streptococcal M protein purified by isoelectric focusing. J. Immunol. 115: 1002—1006, 1975
17) Cunningham, A.J. and Szenberg, A.: Further improvements in the plague technique for detecting single antibody-forming cells. Immunology 14: 599—600, 1968
18) Gell, P.G.H. and Hinde, I.T.: Observations on histology of Arthus reaction and its relation to other known types of skin hypersensitivity. Int. Arch. Allergy Appl. Immunol. 5: 23—46, 1954
19) Uhr, J.W. and Pappenheimer, A.M.Jr.: Delayed hypersensitivity. III. specific desensitization of guinea pig sensitized to protein antigens. J. Exp. Med. 108: 891—904, 1958
20) Okamoto, H., Shoin, S., Koshimura, S. and Shimizu, R.: Studies on the Anticancer and Streptolysin S-Forming Abilities of Hemolytic Streptococci. Japan J. Microbiol. 11: 323—336, 1967
21) Crowle, A.J. and Hu, C.C.: Investigation of the mechanisms by which enhancing antiserum prevents induction of delayed hypersensitivity to protein antigens in mice. J. Allergy 43: 209—223, 1969
22) Baldwin, R.W., Price, M.R. and Robins, R.A.: Inhibition of hepatoma-immune lymph-node cell cytotoxity by tumor-bearer serum, and solubilized hepatoma antigen. Int. J. Cancer 11: 527—535, 1973
23) Beachey, E.H., Stollerman, G.H., Chiang, E.Y., Chiang, T.M., Seyer, J.M. and Kang, A.H.: Purification and properties of M protein extracted from group A streptococci with pepsin: covalent structure of the amino terminal region of type 24 M antigen. J. Exp. Med. 145: 1469—1483, 1977
24) Fischetti, V.A.: Streptococcal M protein extracted by nonionic detergent. II. Analysis of the antibody response to the multiple antigenic determinant of the M-protein molecule. J. Exp. Med. 146: 1108—1123, 1977
25) Weiss, D.W., Bonhag, R.S. and De Ome, K.B.: Protective activity of fractions of tubercle bacilli against isologous tumours in mice. Nature (London) 190: 889—891, 1961
26) Alexander, P. and Evans, R.: Endotoxin and double stranded RNA render macrophages cytotoxin. Nature, New Biol. 232: 76—78, 1971
27) Zbar, B., Bernstein, I.D. and Rapp, H.J.: Suppression of tumor growth at the site of infection with living Bacillus Calmette-Guerien. J. Natl. Cancer Inst. 46: 831—839, 1971
28) Kawrylko, E. and Mackaness, G.B.: Immunoprotentiation with BCG. III. Modulation of the Response to a Tumour

Specific Antigen. J. Natl. Cancer Inst. 51: 1677—1682, 1973
29) Juy, D. and Chedid, L.: Comparison between macrophage activation and enhancement of nonspecific resistance to tumous by mycobacterial immunoadjuvants. Pro. Nat. Acad. Sci. USA 72: 4105—4109, 1975
30) Meltzer, M.S., Tucker, R.W., Sanford, K.K. and Leonard, E.J.: Interaction of BCG-activated macrophages with neoplastic and non-neoplastic cell lines in vitro: Quantitation of the cytotoxic reaction by release of tritiated thymidine from prelabelled target cells. J. Natl. Cancer Inst. 54: 1177—1184, 1975
31) Reggiardo, Z. and Shamsuddin, A.K.M.: Granulomagenic activity of serologically active glycolipids from Mycobacterium bovis BCG. Infect. Immun. 14: 1369—1374, 1976
32) Kuttner, A.G. and Lancefield, R.: In infectious antigens and host reactions (S. Mudd, Ed.): 174. W.B. Saunders, Philadelphia, London, Toronto, 1970
33) Scribner, D.J. and Fahrney, D.: Neutrophil receptor for IgG and complement: their roles in the attachment and ingestion phases of phagocytosis. J. Immunol. 116: 892—897, 1976
34) Spiegelberg, H.L.: In Advances in Immunology (F.J. Dixon and H.G. Kunkel, Eds.) 19: 259—294, Academic Press, New York and London, 1974
35) Dienes, L. and Schoenheit, E.W.: Reproduction of tuberculin hypersensitiveness in guinea pigs with various protein substances. Am. Rev. Tuberc. 20: 92—105, 1929
36) Salvin, S,B. and Smith, R.F.: The specificity of allergic reaction I. Delayed versus Arthus Hypersensitivity. J. Exp. Med. 111: 465—483, 1960
37) Mackaness, G.B., Lagrange, P.H., Miller, T.E. and Ishibashi, T.: Feedback inhibition of specifically sensitized lymphcytes. J. Exp. Med. 139: 543—559, 1974
38) Plant, J. and Glynn, A.A.: Genetics of resistance to infection with salmonella typhimurium in mice. J. Infect. Dis. 133: 72—78, 1976
39) Lefford, M.J., Patel, P.J., Poulter, L.W. and Mackaness, G.B.: Induction of cell-mediated immunity to Mycobacterium lepraemurium in susceptible mice. Infect. Immun. 18: 654—659, 1977