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Studies on the immunopotentiating effects of a streptococcal preparation, OK-432

I. ENHANCEMENT OF T CELL-MEDIATED IMMUNE RESPONSES OF MICE

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SUMMARY

The effects of the anti-tumour agent OK-432 on the immune response to hamster erythrocytes (HRBC) and nucleated chicken erythrocytes (CRBC) were studied in inbred SL mice. Mice were treated repeatedly with OK-432 before immunization with erythrocytes in saline. The cytotoxicity of CRBC-primed spleen cells, as demonstrated by ^{51}Cr release from labelled CRBC, was markedly increased by treatment with OK-432. The delayed footpad reaction to CRBC was significantly augmented by treatment with OK-432. These results in mice indicate that OK-432 can enhance the cellular immune responses which require the contribution of T cells. Such an activation of T cells by OK-432 was observed in the humoral immune response to a trinitrophenyl group. Augmentation of anti-hapten antibody production, suggesting the enhancement of helper T cell activity by OK-432, was noticed after immunization with trinitrophenyl conjugated to erythrocytes. Furthermore, this enhancement of helper T cell activity by OK-432 was confirmed by utilizing an adoptive transfer system. These results support the possibility that T cell activation may be one of the important effects of OK-432 as an immunopotentiator.

INTRODUCTION

OK-432 is a preparation made from an attenuated strain of *Streptococcus haemolyticus*, and is now used as one of the non-specific immunopotentiators for immunotherapy in malignant diseases (Okamoto *et al.*, 1967; Natsuume *et al.*, 1976). Despite the amount of work done on the anti-tumour effect of this agent, its mode of action as an immunopotentiator has still to be resolved.

It has been reported that T cells play an important role in host defence mechanisms to tumour growth (Freedman, Cerottini & Brunner, 1972; Grant, Evans & Alexander, 1973). Consequently, the development of a product which is supposedly active as an immunopotentiator involves assaying in a test system specific for cell-mediated immunity, using tumour-bearing animals. However, it would also be useful to assess the effect of a product in a system free from the influence of the tumour itself, in order to evaluate its fundamental effectiveness on the functions of T cells.

In the present communication, we report studies undertaken in normal mice to see whether OK-432 could influence both cellular and humoral immune responses, to which the contribution of T cells is proven, and we suggest that treatment of mice with OK-432 might augment such T cell functions as the direct cytotoxic activity of spleen cells, the delayed footpad reaction and helper activity in antibody production to heterologous erythrocytes.

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MATERIALS AND METHODS

Animals. Female and male SL mice were obtained from the Breeding Unit, University of Kyushu, and used for experiments at 6 weeks of age. Each group consisted of ten animals, with an equal number of both sexes. Experiments were repeated at least twice.

OK-432. OK-432 was supplied by the Chugai Pharmaceutical Co., Tokyo. Details of the preparation have been described previously (Sakurai *et al.*, 1972). Briefly, a low virulent strain, SU, of *Streptococcus haemolyticus* (Group A), was cultivated in meat-infusion broth at 37°C for about 20 hr. The culture was inoculated into 3% yeast extract medium and cultivated at 37°C for 20 hr. The bacteria were collected by centrifugation, washed twice with a chilled physiological saline, and suspended in 5 vol. of Bernheimer's basal medium containing penicillin G in a final concentration of $1{\cdot}6 \times 10^5$ u/ml. The final concentration was made with 5·0 mg of dried organisms per ml. After cultivation at 37°C for 20 min, the suspension was heated at 45°C for 30 min, and then lyophilized after the addition of an equivalent volume of aqueous 1% DL-methionine solution containing $1{\cdot}08 \times 10^5$ u/ml of penicillin G. This lyophilized preparation is designated OK-432.

Treatment with OK-432. OK-432 was suspended in saline at concentrations of 0·01 and 0·1 mg of dry weight per 0·2 ml. In the standard protocol of experiments, mice were injected intraperitoneally with 0·01 or 0·1 mg of OK-432, every other day to a total of fifteen doses. Controls were injected with 0·2 ml of saline instead of the OK-432 suspensions.

Antigens. Hamster erythrocytes (HRBC) were obtained by cardiac puncture from outbred golden hamsters. Chicken erythrocytes (CRBC) were obtained by venous puncture from young adult hens. These erythrocytes were washed three times with saline and used as immunizing antigens and test antigens. Hapten-conjugated erythrocytes were prepared by reacting with 2, 4, 6-trinitrophenyl-benzene sulphonate (TNP), according to Rittenberg & Pratt (1969). TNP-HRBC and TNP-CRBC were used for immunization. For titration of anti-TNP antibody, trinitrophenylated human erythrocytes of type O (TNP-ORBC) were used as a test antigen.

Immunization. In the standard protocol of experiments, 0·2 ml of a 25% (v/v) suspension of HRBC or CRBC were injected intravenously 2 days after the last injection of OK-432. In order to estimate the activities of helper T cells, 0·2 ml of a 25% TNP-HRBC or TNP-CRBC suspension were injected intravenously, 28 days after the primary immunization with HRBC or CRBC.

Procedures for adoptive immunization. These procedures were carried out according to Hamaoka *et al.* (1969). Donors of carrier-primed cells were injected i.p. with 0·05 mg of OK-432, seven times every other day, and injected i.v. with 0·2 ml of 25% HRBC suspensions 2 days after the last injection of OK-432. Donors of hapten-primed spleen cells were immunized with bovine serum albumin (BSA) conjugated with TNP, according to Rittenberg & Amkraut (1966). Each mouse was injected subcutaneously with 0·5 mg of TNP-BSA in Freund's complete adjuvant. Mice were boosted with TNP-BSA, 1 and 2 weeks later. Spleens were removed 28 days after priming with HRBC or TNP-BSA. Spleen cell suspensions were prepared by squeezing in two glass slides in cold RPMI-1640 culture medium (Gibco). Suspensions containing 3×10^7 hapten-primed cells and either 5×10^7 carrier-primed cells or normal cells, were injected i.v. into recipients which were exposed to 600 R, 6 hr before the cell transfer. The recipients were injected i.v. with 0·2 ml of a 25% TNP-HRBC suspension immediately after the cell transfer. Antibody titres to HRBC or TNP were assessed 5 and 8 days later.

Assay of cytotoxic activity. Mice were bled by cutting the femoral artery, and the spleen served as the source of effector cells. Spleens were squeezed in two glass slides in RPMI-1640 medium containing 10% foetal calf serum. The cell suspension was passed through a gauze filter to remove large fragments. Spleen cells were pooled from three mice of each group. The cell suspensions were adjusted to the desired concentration after counting viability using the trypan blue exclusion method. The labelling of erythrocytes with radioactive ^{51}Cr was carried out according to Perlmann & Perlmann (1970). 100 µCi of Na_2CrO_4 were added to 10^8 cells in 3·0 ml of Tris-buffered Hanks' balanced salt solution containing 2·5% foetal calf serum. After incubation at 37°C for 1 hr, the cells were washed three times with Tris-buffered solution. Then, the labelled cells were resuspended in RPMI-1640 medium containing 10% foetal calf serum. 2×10^7 effector cells were mixed with 10^6 labelled erythrocytes in 2·0 ml of RPMI-1640 medium in roller tubes. The tubes were incubated at 37°C for 6 hr in a CO_2 incubator. After centrifugation at 1000 rev/min for 10 min, the radioactivity of the supernatant was assessed by a liquid scintillation counter. Cytotoxicity was expressed as the percentage release of the radioactivity calculated by the following formula: percentage cytotoxicity $= \frac{x-y}{z-y} \times 100$, where x = ct/min released in the presence of effector cells; y = ct/min released spontaneously from target cells; and z = ct/min released from target cells after treatment with distilled water.

Assay of footpad reaction. The footpad test was carried out using the method of Miller, Mackaness & Lagrange (1973). An eliciting dose of 10^8 erythrocytes in a volume of 50 µl was injected into the subcutaneous tissue on the plantar surface of the right hind foot. The left hind footpad was injected with 50 µl of saline. The degree of swelling was measured after 48 hr with a dial thickness gauge. Reactions were expressed as the difference in the thickness between the feet that had received the erythrocyte injection, and those that had received saline. The mean and standard error of these values were determined in each group.

Assay of antibody titres. Blood was obtained by capillary tubes from the retro-orbital venous plexus at various times after immunization. Haemagglutinin (HA) titres against HRBC, CRBC or TNP were assessed by a microtitration method using Cooke's V plates (Cooke Engineering Company, Alexandria, Virginia). HA titres were expressed as $\log_2$, and the mean and standard error were determined in each group. For the examination of 2-mercaptoethanol (2-ME) resistant antibodies, sera

were diluted with saline containing 2-ME so as to obtain 0·1 M final concentration, and incubated for 30 min at 37°C before the addition of test antigens.

Statistics. For comparison between groups the Student's *t*-test was used.

RESULTS

Cytotoxic activity of spleen cells

The CRBC suspension was injected into mice treated with OK-432 and those treated with saline, and the cytotoxic activity of spleen cells against CRBC was asssesed on day 0, 7, 14 and 21 after the injection of CRBC. As shown in Fig. 1, cytotoxicity was approximately 57% on day 7, and 44% on day 14, in mice which received treatment with OK-432. The cytotoxicity declined to 15% on day 21. No apparent difference was noticed between mice treated with 0·01 mg and 0·1 mg of OK-432. In contrast, the cytotoxic activity of saline-treated controls was only 15% on day 7, and negligible on days 14 and 21. These results indicate that OK-432 could enhance the cytotoxic activity of spleen cells in mice.

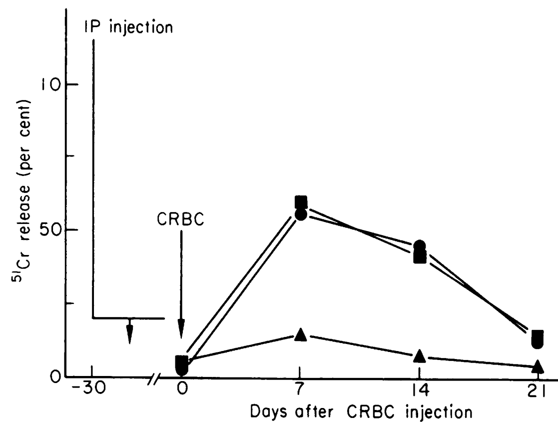

FIG. 1. Effect of treatment with OK-432 on the direct cytotoxic activity of spleen cells aginst CRBC. Mice were injected i.p. with (■—■) 0·01 mg, (●—●) 0·1 mg dry weight of OK-432, or (▲—▲) saline, every other day to a total of fifteen doses. 0·2 ml of a 25% CRBC suspension in saline was injected intravenously 2 days after the last injection of OK-432. Direct cytotoxicity of spleen cells against CRBC was measured on various days after CRBC injection. Each value represents the mean percentage cytotoxicity of ten animals in each group.

Delayed footpad reaction

The CRBC suspension was injected into mice treated with OK-432 and those treated with saline, and the footpad reaction was elicited on day 0, 7, 14, and 21 after the injection of CRBC. As shown in Fig. 2, a rather strong delayed reaction was evoked on day 7, even in saline-treated controls. However, the reactions on day 14 and 21 were already diminished. In contrast, a highly positive reaction was found in mice treated with OK-432 throughout the experiment. Treatment with 0·1 mg of OK-432 gave a statistically significant difference, as compared to controls ($P<0{\cdot}01$ on day 7 and 14, $P<0{\cdot}05$ on day 21). A similar pattern of augmentation was noticed in mice treated with 0·01 mg of OK-432, although the degree of augmentation was lower than in those treated with 0·1 mg. Thus, OK-432 was found to have an augmenting effect on the delayed footpad reaction in mice.

Antibody production to CRBC, HRBC and TNP

OK-432-treated mice and controls were primarily immunized with CRBC, followed by TNP-CRBC 28 days later. Antibody titres to CRBC were measured on day 0, 4, 8 and 14, respectively. Anti-TNP antibody titres were measured on day 28, 32 and 36, respectively. As shown in Fig. 3, treatment with 0·1 mg of OK-432 augmented antibody production to CRBC. A statistically significant difference was

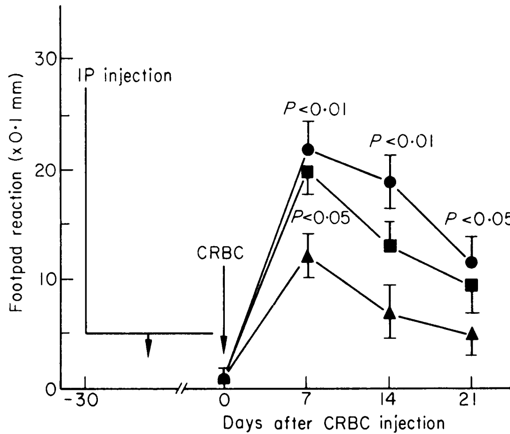

FIG. 2. Effect of treatment with OK-432 on footpad reaction against CRBC. Mice received treatment with OK-432 and immunization with CRBC as described in Fig. 1. (●—●) OK-432, 0·1 mg; (■—■) OK-432, 0·01 mg; and (▲—▲) saline, 0·2 ml. The footpad test was performed on various days after CRBC injection. Each value represents the mean ± s.e. of the values of ten animals in each group.

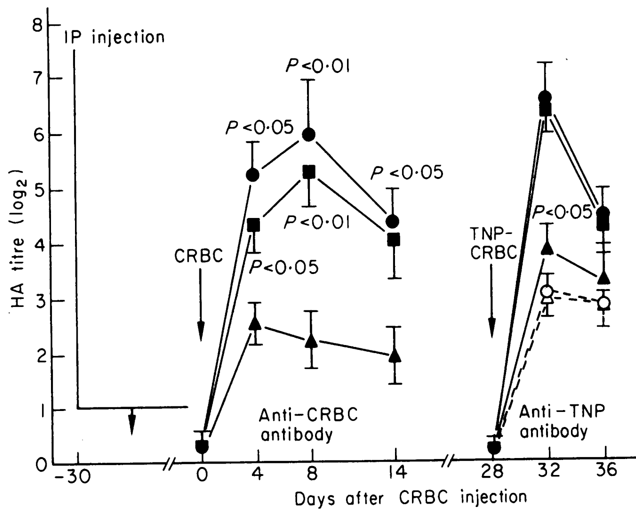

FIG. 3. Effect of treatment with OK-432 on antibody production to CRBC and TNP-CRBC. 0·2 ml of a 25% CRBC suspension in saline was injected i.v. and anti-CRBC antibody titres were measured on days 0, 4, 8 and 14 after CRBC injection. 0·2 ml of a 25% TNP-CRBC suspension was injected 28 days after the CRBC injection, and anti-TNP antibody titres were measured on day 28, 32 and 36 after the CRBC injection. Each value represents the mean ± s.e. of HA titres of ten animals in each group. OK-432, 0·1 mg (●—●, ○—○), 0·01 mg (■—■) and saline (▲—▲). (○—○) and (△—△): Titres in mice which did not receive the prior priming with CRBC.

detected between mice treated with 0·1 mg of OK-432, and controls ($P<0{\cdot}05$ on day 4 and 14, $P<0{\cdot}01$ on day 8). Treatment with 0·01 mg of OK-432 also gave rise to a significant increase in antibody production to CRBC, although this was less than the effect of 0·1 mg ($P<0{\cdot}05$ on day 4, $P<0{\cdot}01$ on day 8). Antibody production to TNP was enhanced by treatment with OK-432, as compared to control mice previously primed with CRBC ($P<0{\cdot}05$ on day 32). However, OK-432 could not enhance the anti-TNP antibody response to the immunization with TNP-CRBC in those mice not previously primed with CRBC, since the anti-TNP antibody titre in mice treated with OK-432 was not significantly different from that in mice treated with saline. These results indicate that OK-432 had an enhancing

effect on the antibody response of mice against immunization with a heterologous erythrocyte. In addition, it was found that OK-432 enhanced the effect of previous priming with a carrier on antibody response to immunization with the hapten-carrier conjugate, i.e. the carrier effect.

The results obtained using HRBC and TNP-HRBC were essentially the same as those obtained using CRBC and TNP-CRBC (Fig. 4).

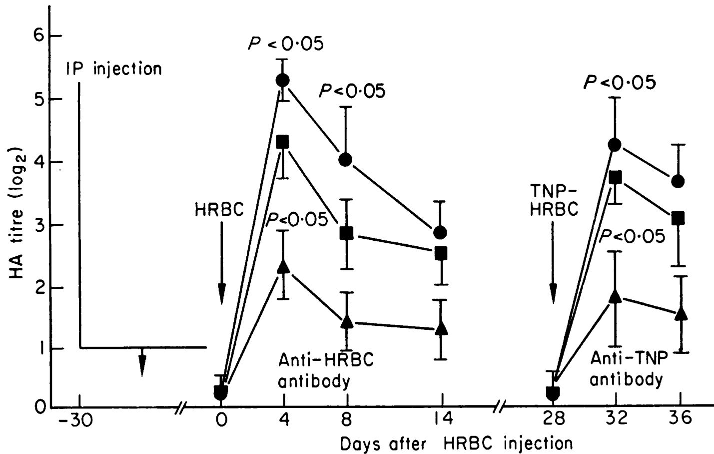

FIG. 4. Effect of treatment with OK-432 treatment on antibody production to HRBC and TNP-HRBC. Antibody response was assayed in a manner similar to that described in Fig. 3. (●—●) OK-432, 0·1 mg; (■—■) OK-432, 0·01 mg; and (▲—▲) saline, 0·2 ml.

Influence of treatment with OK-432 on the helper activity of T cells

In order to obtain further information as to the precise effect of OK-432 on the co-operation of hapten-specific B cells and carrier-specific T cells, an adoptive transfer system was employed. When normal spleen cells were transferred together with hapten-primed cells, antibodies to HRBC or TNP were not detected in the recipients, as shown in group III of Table 1. When spleen cells of mice primed with HRBC, but not treated with OK-432, were transferred together with hapten-primed cells, a low titre of anti-TNP antibody was found on day 8 (group II). In contrast, 2-ME-sensitive antibody to TNP was found on day 5, and an appreciable titre of 2-ME resistant anti-TNP antibody became detectable on day 8, when carrier-primed cells were obtained from mice treated with OK-432 (group I). The 2-ME resistant anti-TNP antibody titre of group I was significantly higher than that of group II ($P<0.05$). These results indicate that the carrier effect by T cells, i.e. helper T cell activity, could be enhanced by treatment with OK-432.

DISCUSSION

It has been reported previously that OK-432 exerts an anti-tumour effect against transplantable tumours in experimental animals (Ohta, 1957; Okamoto *et al.*, 1967; Sakurai *et al.*, 1972; Kaibara *et al.*, 1972), and suppresses the development of some mouse leukaemias (Suzuki, Yamamoto & Ogawa, 1975; Aoki *et al.*, 1976). The anti-tumour effect of this agent had been attributed to a direct cytotoxic effect on tumour cells (Sakurai *et al.*, 1972) and to an inhibitory effect on the nucleic acid synthesis of tumour cells (Ono *et al.*, 1973). Later, OK-432 was found to have an activating effect on defence mechanisms of animals against tumour growth, as pre-treatment with this agent resulted in the prolongation of the life-span after the inoculation of tumour cells (Kimura *et al.*, 1974). Recently, it has been demonstrated that OK-432 can activate macrophages (Ishii *et al.*, 1976) and serum complement components, presumably via the alternative pathway (Kondo *et al.*, 1975; Natsuume *et al.*, 1976). Antibody production in mice against gross cell surface antigens and the cell surface differentiation antigen was found to be augmented

TABLE 1. Effect of OK-432 treatment on helper T cell activity

	Carrier-primed spleen cells		Hapten-primed spleen cells	HA titres ($\log_2$)†			
				Day 5		Day 8	
Group	OK-432*	Carrier	Hapten-carrier	Anti-HRBC 2-ME-R‡ Net	Anti-TNP 2-ME-R Net	Anti-HRBC 2-ME-R Net	Anti-TNP 2-ME-R Net
I	Yes	HRBC	TNP-BSA	0	0	1·64±0·79	2·35±0·45§
				3·18±0·76§	1·78±0·67	3·92±0·76	3·86±0·99
II	No	HRBC	TNP-BSA	0	0	1·28±0·62	0·71±0·34
				1·06±0·42		2·57±0·81	1·14±0·40
III	No	No	TNP-BSA	0	0	0	0

* Treatment of carrier-primed cell donors with i.p. injection with 0·1 mg of OK-432, every other day to a total of fifteen doses.

† HA titres in recipient mice after immunization with TNP-HRBC. Each value represents the mean ± s.e. of HA titres of ten animals.

‡ 2-mercaptoethanol-resistant antibody titres.

§ $P < 0{\cdot}05$.

by treatment with OK-432 (Aoki *et al.*, 1975). These findings suggest that OK-432 may have an enhancing effect on the immune resistance of the host to the tumour.

On the other hand, it is widely accepted that T cells may also play an important role in the immune surveillance mechanism, i.e. cell-mediated immunity, including discrimination of tumour cells from normal cells and induction of immune resistance against tumour growth. The activation of specific B cells either requires, or is markedly enhanced, by the activity of T cells (Kitagawa *et al.*, 1974). Thus, it seems essential to understand the effects of OK-432 on the functions of T cells for its successful use as an immunopotentiator. As yet, however, little is known about this.

The experimental results in normal mice presented here clearly show that OK-432 has enhancing effects on both cellular and humoral immune responses to which the contribution of T cells is proven. CRBC have been used as target cells for antibody-dependent cell-mediated cytotoxicity (ADCC) (Perlmann & Perlmann, 1970). However, Kubo *et al.* (1977) have found that CRBC can induce direct cytotoxicity in spleen cells after intravenous or intraperitoneal immunization in mice. In their report, such a direct cytotoxicity was found to reside in a θ-positive glass-non-adherent population of spleen cells. In the present study, OK-432 was able to augment direct cytotoxicity of spleen cells against CRBC, which suggested that OK-432 might augment T cell-dependent cytotoxicity, although we did not use anti-θ antiserum.

The footpad test devised by Gray & Jennings (1955) is currently more widely used than any other kind of test for detecting delayed-type hypersensitivity (DTH) in mice (Crowle, 1975). The delayed footpad reaction to CRBC was clearly augmented by treatment with OK-432. Since DTH in mice has been shown to be a function of T cells (Pritchard & Micklem, 1972), OK-432 may therefore have an enhancing effect on the T cell-mediated immune response. DTH against protein antigens has been demonstrated to include two different types. One is the tuberculin type and the other is the Jones–Mote type. The augmented footpad reaction by OK-432 was seen when apparent antibody production to CRBC was present, as shown in Fig. 3. OK-432 appears to augment a stable form of DTH which we suggest, is comparable to the tuberculin type-hypersensitivity seen in the guinea-pig (Ohmichi *et al.*, 1976).

Treatment with OK-432 augmented antibody production to CRBC or HRBC. Moreover, antibody production to TNP was augmented by OK-432 in mice primed with CRBC or HRBC (Figs. 3 & 4). This augmentation was observed only when mice had received prior carrier priming (Fig. 3). It has been reported that the hapten-specific antibody response to immunization with a hapten-carrier conjugate

can be enhanced by previous priming with the same carrier (Rajewsky *et al.*, 1969); this effect is termed the carrier effect. Therefore, OK-432 would appear to further enhance the carrier effect. This effect has been attributed to the co-operation between hapten-specific B cells and carrier-specific helper T cells (Mitchison, 1971; Kettman & Dutton, 1971; Katz & Benacerraf; 1972). The observed enhancement of the carrier effect was shown to be related to the enhancement of helper activity by OK-432, using the adoptive transfer system (Table 1). Irradiated mice transferred with TNP-BSA and HRBC-primed spleen cells were capable of provoking anti-TNP antibody production against immunization with TNP-HRBC. This state was clearly enhanced by treatment with OK-432. It is worth noting that both 2-ME-sensitive and 2-ME-resistant antibody production was augmented by treatment with OK-432, since it has been reported that primed T cells can help IgM production and the shift to IgG (Doria *et al.*, 1977).

Recently, helper cell induction to either soluble or particulate antigen has been shown to require the co-operation of T cells and macrophages (Erb & Feldmann, 1975). Because OK-432 has been found to activate macrophages (Ishii *et al.*, 1976), the possibility that the enhancement of helper T cell activity is a secondary phenomenon which may follow the activation of macrophages by OK-432 cannot be ruled out. In this connection, Askonas & Roelants (1974) and Unanue & Katz (1973) have shown that transferred macrophages enhance antibody synthesis to the hapten only when macrophages have been previously stimulated *in vivo* with the complex used for immunization; a mixture of macrophages stimulated with the hapten or with the carrier was ineffective. Thus, in our experiments it is more likely that OK-432 would exert a direct effect on helper cell activity and not on the macrophages.

From these results, it is suggested that OK-432 has an enhancing effect on various T cell functions in immune reactions, such as direct cytotoxic activity of spleen cells, delayed footpad reaction and helper cell activity. Kondo *et al.* (1975) reported that OK-432 could activate T cells responsible for the stimulation by phytohaemagglutinin (PHA) in humans. This evidence is consistent with present results.

BCG (Bacillus Calmette–Guérin), a known immunopotentiator, has been found to exert an enhancing effect on helper T cell activity in mice (Kitamura *et al.* 1976), and on both the proportion and the function of T cells in humans (Torisu *et al.*, 1976). It is widely accepted that T cells play important roles in both the cellular and humoral immune responses to many antigens. Therefore, T cell activation may be one of the important effects of OK-432 as an immunopotentiator.

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