[Chem. Pharm. Bull. 27(1) 166—175 (1979)]

UDC 615.277.3.036.076.7 : 576.851.2.093

Cytolytic Mechanism of Attenuated Preparation (OK-432) of *Streptococcus Haemolyticus* against Ascites Hepatoma, AH 130 and AH 41 C Cells[1)]

Taro Ogiso, Hiroyuki Masuda, and Masaya Seko

Gifu College of Pharmacy[2)]

(Received August 2, 1978)

In order to gain insight into the mechanisms by which OK-432 (a lyophilized preparation of an attenuated strain of *Streptococcus haemolyticus*) treatment lyzed tumor cells, the *in vivo* effect of the agent on some enzymes, cellular components and surface properties of ascites hepatoma, AH 130 and AH 41 C cells, was studied in comparison with the effect of 5-fluorouracil (5FU) and cyclophosphamide (CP) on these parameters. Administration of OK-432 (0.5 mg/rat/day) to AH 130 cell-bearing rats for 4 days was found to enhance leakage of intracellular potassium and calcium, cause a decrease in hexokinase activity, cause a decrease in saturated fatty acids but a significant increase in cholesterol, unsaturated fatty acids of total phospholipids and 2,4,6-trinitrobenzenesulfonate binding to phosphatidylethanolamine as compared with the control cells. OK-432 administered for 2 days to AH 41 C cell-bearing rats also enhanced the leakage of the intracellular cations and the proportion of arachidonic acid in total phospholipids. On the other hand, significant alterations of these parameters in the cells were not demonstrated by 5FU and CP treatments. OK-432 treatment is considered to have an important influence on the lipid metabolism of the cells resulting in an increase in membrane fluidity which is related to leakage of essential components and hexokinase, consequently the leakage appears to inhibit the cell growth.

Keywords——streptococcal preparation; cytolytic action against ascites hepatoma; ascites hepatoma; changes in components of ascites hepatoma; cytolytic activity of streptococcal preparation; leakage of components from ascites hepatoma

Since Busch presented his noteworthy report in 1868 that a human malignant tumor was suppressed when the patient had erysipelas,[3)] many investigators have tried to make use of hemolytic streptococci for finding an approach to the treatment of cancer. OK-432, a lyophilized preparation of an attenuated strain of *Streptococcus haemolyticus*, was found to suppress growth of some lines of transplantable tumors[4)] through a host mediated immune mechanism such as activation of macrophages.[5)] It also exerts a direct cytocidal action on tumor cells, attributed to its inhibitory effect on RNA synthesis in tumor cells.[6)] However, the biochemical mechanism of the lytic effect of the agent on tumor cells has been only partially clarified. In an attempt to clarify the mechanism of the action, we examined the *in vivo* effect of OK-432 on the enzymes, some components and surface electrical properties of ascites hepatoma, AH 130 and AH 41 C cells, as well as 1-anilinonaphthalene 8-sulfonate (ANS) and 2,4,6-trinitrobenzenesulfonate (TNBS) bindings to both the cells. A comparison

1) This work was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April, 1978.
2) Location: *Mitahora, Higashi-5-6-1, Gifu.*
3) W. Busch, *Berlin Klin. Wschr.*, **5**, 137 (1868).
4) *a*) Y. Sakurai, S. Tsukagoshi, H. Satoh, T. Akiba, S. Suzuki, and Y. Akiba, *Cancer Chemother. Rep.*, **56**, 9 (1972); *b*) H. Okamoto, M. Minami, S. Shoin, S. Koshimura, and R. Shimizu, *Zikken Igaku Zasshi*, **36**, 175 (1966).
5) Y. Ishii, H. Yamaoka, K. Toh, and K. Kikuchi, *Gann*, **67**, 115 (1976); A. Chaffar, R.T. Cullen, N. Dunbar, and M.F.A. Woodruff, *Brit. J. Cancer*, **29**, 199 (1974); R.P. Cleveland, M.S. Meltzer, and B. Zbar, *J. Nat. Cancer Inst.*, **52**, 1887 (1974); J.B. Hibbs, L.H. Lambert, and J.S. Remington, *Nature New Biol. London*, **235**, 48 (1972).
6) T. Ono, S. Kurata, K. Wakabayashi, Y. Sugawara, M. Saito, and H. Ogawa, *Gann*, **64**, 59 (1973).

of the effects of OK-432 on the tumor cells with those of 5-fluorouracil (5FU) and cyclophosphamide (CP) was also performed.

Experimental

Materials——OK-432 was obtained from Chugai Seiyaku Co., Ltd. 5FU and CP were a gift from Kyowa Hakko Co., Ltd. and Shionogi Seiyaku Co., Ltd., respectively. ANS and TNBS were purchased from Tokyo Kasei Kogyo Co., Ltd. Rat ascites hepatoma, AH 130 and AH 41 C cells, maintained by weekly transplantation of 0.3 ml of undiluted ascites fluid into Donryu rat, were used for this experiment.

Animals and Treatment——Male Donryu rats weighing 95—100 g, maintained on MF diets (Oriental Yeast Co., Ltd.) for 3—4 days prior to the experiments, were divided at random into 2 groups, each consisting of 5—6 rats. A) Rats were inoculated intraperitoneally with 3×10^6 cells of AH 130 (or AH 41 C) on day 1 and treated for 4 (or 2) successive days from day 7 to 10 (or on day 9 and 10) with daily intraperitoneal (*i.p.*) injections of saline (control group). B) Animals were inoculated with AH 130 or AH 41 C cells as described in A) and treated for 4 successive days from day 7 to 10 with single daily *i.p.* injections of OK-432 (0.5 mg/rat/day) (OK-432 group). In the experiments involving AH 41 C cells the injection of OK-432 was done for 2 days, on day 9 and 10. On day 11 the peritoneal fluids of the rats were removed by syringes. The fluids mixed with the same volume of Eagle MEM containing 10% fetal bovine serum were transferred to flat-bottomed glass tubes and incubated at 37° for 20 min. The cells adhering to the glass surface were removed and the same treatment was further carried out two times. The non-adherent cells obtained were washed several times with cold saline. The cells obtained from the rats dosed with 5FU or CP were washed with saline without removal of adherent cells. The doses of 5FU (30 mg/kg/day) and CP (9.9 mg/kg/day) to rats were done from day 7 to 10 intraperitoneally. The cell suspension was evaluated for viability by the trypan blue exclusion reaction. Morphologically, more than 95% of the mononuclear cells present in these suspensions were tumor cells.

Measurements of Total Number of Tumor Cells——The number of tumor cells was counted using a Bürker Türk counting chamber.

Determination of Sialic Acid——The cells washed with saline were hydrolyzed at 80° in 0.1 N H_2SO_4 in evacuated, sealed tubes for 1 hr. Sialic acid was determined by the procedure of Warren[7] with N-acetylneuraminic acid as a standard.

Determination of Amino Sugar——The washed cells were hydrolyzed at 105° with constant-boiling HCl in evacuated, sealed tubes for 6 hr. Amino sugars were estimated by the Elson-Morgan reaction[8] with glucosamine as a standard.

Measurements of Electrophoretic Mobility——The electrophoretic mobility (in μ/sec/V/cm) of the cells suspended in 0.25 M sucrose was measured using a Zeta-meter (Zeta-meter Inc., New York) at 25°.

Determination of Potassium and Calcium——For the determination of potassium and calcium, the washed cells were mixed with 2.5 volumes of 7.5 mM lanthanum chloride and left standing in ice for 20 min. To 5 volumes of the mixture 1 volume of 30% trichloroacetic acid was added and mixed for 1 min by a Potter homogenizer, followed by centrifugation. The amount of potassium and calcium in the supernatant was estimated with a Hitachi atomic absorption spectrophotometer at 766.5 and 422.7 nm, respectively.

Determination of Protein——Protein concentration was determined by the procedure described by Lowry, *et al.*[9] with bovine serum albumin as a standard.

Determination of Cholesterol——The content of total cholesterol in the cells was determined by the method of Zak-Henly[10] with pure cholesterol as a standard.

Separation of Lipids and Fatty Acid Analyses——Extraction of the cell lipids and separation of the neutral lipids and phospholipids were done by the method of Colbeau *et al.*,[11] using a column of silicic acid–Celite 545 (2:1, by weight). Separation of phospholipid species was performed by thin-layer chromatography (TLC) using a 0.25 mm-thick layer of Kieselgel 60HR (E. Merck) and a solvent mixture composed of chloroform–methanol–H_2O (65:25:4, by volume). The fatty acids of lipids, following evaporation *in vacuo*, were methylated by incubating with 5% H_2SO_4 in methanol for 72 hr (36 hr for neutral lipids) at 40°.[12] The methylated acids were analyzed by gas–liquid chromatography (GLC) on a Shimadzu gas chromatograph Model GC-4BM apparatus with a hydrogen flame ionization detector, using a 3 mm × 2 m stainless steel column packed with 15% polyethylene glycol succinate polyester on 60—80 mesh chromosorb W.

7) L. Warren, *J. Biol. Chem.*, **234**, 1971 (1959).
8) R. Belcher, A.J. Nutten, and C.M. Sambrook, *Analyst*, **79**, 201 (1954).
9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) B. Zak, *Am. J. Clin. Path.*, **27**, 583 (1957); A.A. Henly, *Analyst*, **82**, 286 (1957).
11) A. Colbeau, J. Nachbaur, and P.M. Vignais, *Biochim. Biophys. Acta*, **249**, 462 (1971).
12) T.W. Keenan and D.J. Morrĕ, *Biochemistry*, **9**, 19 (1970).

TNBS Binding to Aminophospholipids——TNBS binding to phosphatidylethanolamine was carried out according to the method of Bonsall and Hunt[13] with a slight modification, as described in a previous paper.[14]

Determination of Phospholipid Phosphorus——The phospholipid phosphorous content was estimated by the method of Ames.[15]

Electron Microscopy——Tumor cells, untreated or treated with OK-432, were fixed with 1.5% glutaraldehyde in isotonic phosphate buffer, pH 7.2. The cells were washed 4 times with the same buffer and dried with increasing concentrations of aceton (60 to 100%). The specimens were coated at continuously varying angles with gold and viewed with a Nihon Denshi scanning electron microscope, Model JEM-100B.

Measurements of ANS-cell Fluorescence——Two ml of the cell suspensions (0.5 mg of protein/ml of isotonic phosphate buffer, pH 7.0) was mixed with an equal volume of 50 μM ANS dissolved in the same buffer in a quartz cell of 1.0 cm-optical path and after 1 min the fluorescence of ANS associated with the membrane was measured in a Shimadzu RF-501 recording spectrofluorophotometer at 20°. The excitation wavelength was held constant at 380 nm and emission wavelength was 480 nm.

Assays of Adenosine Triphosphatase (ATPase) and Hexokinase Activities——For the assay of ATPase activity, the washed cells were homogenized in 5 volumes of 10 mM Tris-HCl buffer, pH 7.4, for 2 min under chilling using a Potter homogenizer. The total volume (1.0 ml) contained 3 mM Tris-ATP (Na^+ was eliminated with an Amberlite IR-120 column), 5 mM $MgCl_2$, 140 mM KCl, 20 mM Tris-HCl buffer, pH 7.4, and 0.5 ml of the cell homogenate (5—8 mg protein/ml). The reaction was terminated by addition of 1 ml of 10% trichloroacetic acid. After centrifugation, inorganic phosphate in the supernatant was determined according to the method described by Fiske and SabbaRow.[16] For the assay of hexokinase activity, the cells were mixed with an equal volume of 0.4% Triton X-100 solution and left standing in ice for 30 min, followed by centrifugation. A mixture consisting of 0.5 ml of 200 mM Tris-HCl buffer, pH 7.0, 0.1 ml of 200 mM $MgCl_2$, 0.5 ml of 10 mM glucose, 0.3 ml of water, 0.1 ml of 1 M NaF, 0.5 ml of 20 mM ATP and 1.0 ml of the supernatant was incubated for 20 min at 37° and the reaction was terminated by the addition of 3 ml each of 0.3 N $Ba(OH)_2$ and 5% $ZnSO_4$. Glucose in the filtrate was assayed according to the method of Somogyi-Nelson.[17]

Results

In Vivo Cytolytic Activity of OK-432 against AH 130 and AH 41 C Cells

Cytolytic activity towards the tumor cells was examined by measuring the number of the cells after administration of OK-432 (0.5 mg/rat/day) from day 2 to 8. As shown in Fig. 1, the number of AH 130 cells decreased only slightly by the treatment for 4 successive days (at 6 days) and to about one third after doses for 7 successive days (at 9 days). This indicates that OK-432 has a cytolytic activity, but is not potent, to AH 130 cells. On the other hand, the number of AH 41 C cells significantly declined after dosing the agent for 3 days (at 5 days) and the cells nearly disappeared after its administration for 7 successive days. These data agree with that reported by Sakurai *et al.*[4a]

In order to gain insight into the mechanisms of tumor cytolysis with OK-432, the changes in the cellular components and enzymes were examined just before the cell decay. As a result, OK-432 was given to AH 130 and AH 41 C cell-bearing rats for 4 and 2 days, respectively.

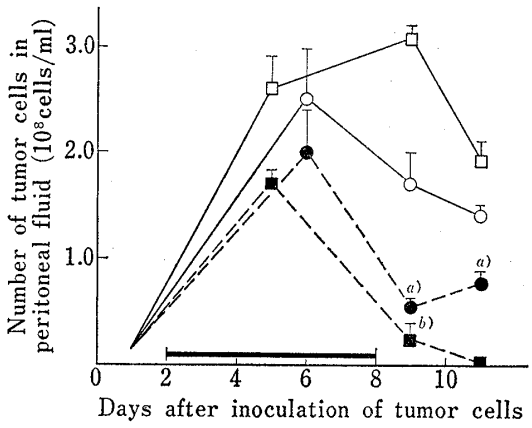

Fig. 1. *In Vivo* Cytolytic Activity of OK-432 against the Tumor Cells

0.3 ml (10^{11} cells/ml) of tumor cell suspension was inoculated on day 1 and OK-432 (0.5mg/rat/day) was administered from day 2 to day 8, as presented with a bar. Each point is the mean of 4 rats ±S.E.
a) $p<0.05$, *b*) $p<0.001$ AH 130 rats (○); AH 130 rats treated with OK-432 (●); AH 41 C rats (□); AH 41 C rats treated with OK-432 (■).

13) R.W. Bonsall and S. Hunt, *Biochim. Biophys. Acta*, **249**, 281 (1971).
14) T. Ogiso, K. Kuhara, N. Noda, and Y. Kato, *Chem. Pharm. Bull.* (Tokyo), **24**, 1893 (1976).
15) B.N. Ames, "Methods in Enzymology," Vol. 8, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York and London, 1966, p. 115.
16) C.H. Fiske and Y. SabbaRow, *J. Biol. Chem.*, **66**, 375 (1925).
17) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

Scanning Electron Microscopic Observations

An aliquot (10^7 cells/ml) of AH 130 cells suspended in Eagle MEM was incubated with high level of OK-432 (0.5 mg/ml) for 3 hr at 37° in order to find alterations of the membrane. Fig. 2 shows typical scanning electron micrographs of AH 130 cells. The agent induced visual shape changes in the cells; the treatment appears to result in the aggregation of the membrane particles, indicating a drastic perturbation of the membrane. AH 130 and AH 41 C cells from the rats treated *in vivo* with OK-432 (0.5 mg/rat/day) for 4 days had a similarly morphological change, but not so significant as the *in vitro* experiment.

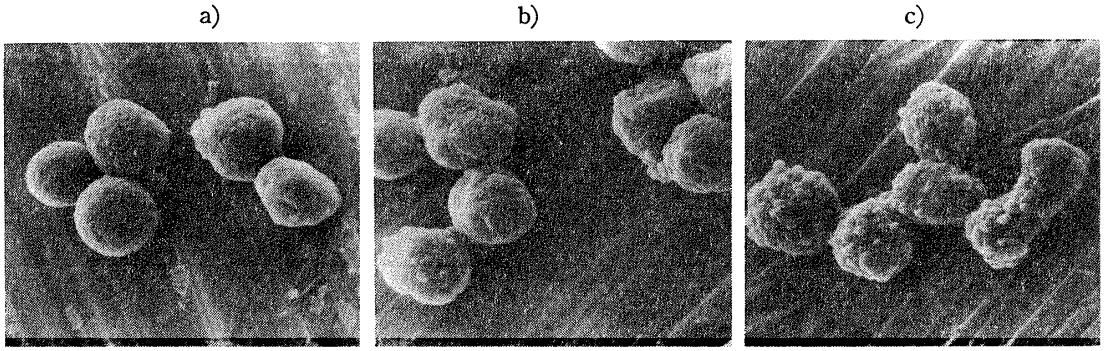

Fig. 2. Scanning Electron Micrographs of AH 130 Cells treated with OK-432

a) native tumor cells; b) tumor cells (10^7 cells/ml) incubated in Eagle MEM for 3 hr at 37°; c) tumor cells (10^7 cells/ml) incubated with OK-432 (0.5 mg/ml) in Eagle MEM for 3 hr at 37°. Magnification 3000×.

Effect of OK-432 on AH 130 Cells

1) Total Number, Components and Enzyme Activities of the Cells——OK-432 (0.5 mg/rat/day) was administered from day 7 to 10 after inoculation of the tumor cells, and on day 11 the treated and untreated (control) cells were collected. No significant difference could be observed between the numbers of the cells in the control ($1.73\pm0.10, \times 10^8$/ml) and OK-432 ($1.67\pm0.15, \times 10^8$/ml) groups on day 11. As shown in Table I, the contents of sialic acid and amino sugar were little changed by administration of OK-432. The amount of calcium and potassium of the cells was markedly decreased by the treatment, probably due to leakage of the components from the cells. The activity of ATPase, a membrane-bound enzyme of the cells, was not affected by administration of the agent, while the activity of hexokinase, a regulatory enzyme of glycolysis, was significantly decreased by the treatment (approximately 19% decrease compared to the control). The content of phospholipid phosphorus in the cells was not extremely decreased by this treatment. The level of cholesterol was

Table I. Effect of OK-432 on Cellular Components and Enzyme Activities of AH 130 Cells

Component	Control	OK-432
Sialic acid[a)]	7.02 ±0.28	6.18 ±0.61
Amino Sugar[b)]	9.90 ±0.69	9.49 ±0.60
Phospholipid phosphorus[c)]	0.128±0.013	0.104±0.014
Cholesterol[d)]	2.39 ±0.21	3.57 ±0.32[g)]
Potassium[d)]	24.86 ±1.62	17.34 ±1.01[g)]
Calcium[d)]	0.19 ±0.02	0.16 ±0.01[h)]
Hexokinase[e)]	22.14 ±1.94	17.95 ±1.10[h)]
ATPase[f)]	4.95 ±0.46	4.95 ±0.49

OK-432 (0.5 mg/rat/day) was administered from day 7 to day 10 after inoculation of the tumor cells. Each value represents the mean ±S.D.
a) nmol N-acetylneuraminic acid/mg protein; *b*) μg glucosamine/mg protein; *c*) μmol P/mg protein; *d*) μg/mg protein; *e*) mmol glucose/mg protein/min; *f*) μmol P/mg protein/min. *g*) $p<0.01$; *h*) $p<0.05$.

much more increased in the cells treated with OK-432 than in the control, suggesting that the increased level of cholesterol may take part in diminishing the degree of membrane fluidity which was increased owing to the altered composition of the fatty acids. No changes in the surface electrical properties (Zeta potential) of the cells were found after administration of OK-432 (control cells; 1.60 μ/sec/V/cm, treated cells; 1.63 μ/sec/V/cm).

2) Binding of Hydrophobic Probes to the Cells——TNBS has been used to study the outside of biological membranes[18] and it easily binds to amine groups of phospholipids and proteins on the membrane.[13] Phospholipids of the cells of both groups were separated by TLC and the extent of reaction of TNBS with phosphatidylethanolamine was estimated by ninhydrin reaction. The results shown in Table II indicate that treatment of the cells with OK-432 increased the amount of the phospholipid which reacted with the reagent by 24% as compared with the control. The increase in the accessibility of the phospholipid to TNBS may be due to extreme alterations of the membrane permeability and organization. However, an increase in ANS-fluorescence intensity was not significant in the cells treated with OK-432.

Table II. Binding of Hydrophobic Probes to AH 130 Cell Components

	Control	OK-432
ANS-fluorescence intensity/mg protein	1.31± 0.15	1.47± 0.23
TNBS binding to phosphatidylethanolamine (%)	69.27±11.81	85.94±10.22[a]

Each value represents the mean ±S.D. *a*) $p < 0.05$.
Experimental conditions are described in text.

3) Fatty Acid Composition of Neutral Lipids and Phospholipids of the Cells——The fatty acid composition of the neutral lipids and phospholipids of the cells is shown in Figs. 3 and 4, respectively. The fatty acid composition of neutral lipids shown in Fig. 3 indicates that after dosing OK-432 for 4 days a clear per cent increase in linoleic (112%) and arachidonic (67%) acids was induced, while stearic acid content declined by 14% as compared with that of normal AH 130 cells. The fatty acid composition of the phospholipids is shown in Fig. 4. A significant increase in arachidonic (55%) and linoleic (23%) acids and a slight decline in

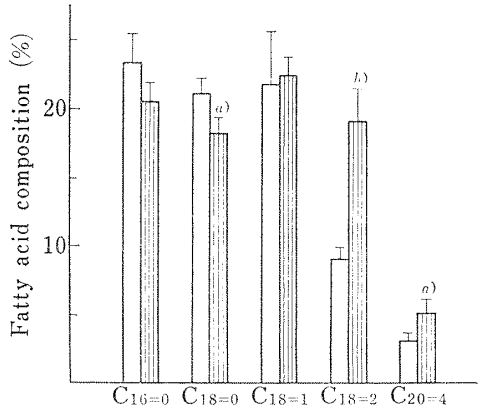

Fig. 3. Fatty Acid Composition of Neutral Lipids AH 130 Cells treated with OK-432

Only the major acids are given. Each value represents the mean of 4 rats ±S.D.
a) $p<0.05$; *b*) $p<0.001$; ▭ control; ▤ OK-432.

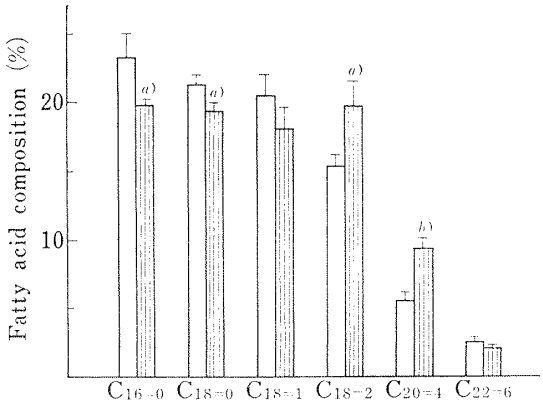

Fig. 4. Fatty Acid Composition of Phospholipids of AH 130 Cells treated with OK-432

Only the major acids are given. Each value represents the mean ±S.D.
a) $p<0.05$; *b*) $p<0.01$; ▭ control; ▤ OK-432.

18) J. van Steveninck, R.I. Weed, and A. Rothstein, *J. Gen. Physiol.*, **48**, 617 (1965); H.C. Berg, *Biochim. Biophys. Acta*, **183**, 65 (1969).

palmitic and stearic acids in phospholipids were observed after injections of OK-432. Thus, OK-432 treatment appears to have a significant influence on lipid metabolism of the cells and the alterations of fatty acid composition probably affect the physical state of the membrane.

4) **Fatty Acid Composition of Phosphatidylethanolamine and Phosphatidylcholine**——The percentage composition of fatty acids in these phospholipids is shown in Table III, as compared with the control. The cells treated with OK-432 reduced the proportion of oleic and palmitic acids of phosphatidylethanolamine by 24 and 21% respectively, and significantly increased the content of arachidonic acid by 75%. The result roughly agreed with the data obtained with total phospholipids, except stearic and linoleic acids; the content of the former was not altered but the latter was decreased in phosphatidylethanolamine. No difference in the fatty acid composition of phosphatidylcholine was shown between the control and OK-432-treated rats.

Table III. Fatty Acid Composition of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) of AH 130 Cells Treated with OK-432

Fatty acid		Control (%)	OK-432 (%)
$C_{16=0}$	PC	31.16±2.38	33.88±2.63
	PE	9.50±0.52	7.48±0.60[a]
$C_{18=0}$	PC	17.93±1.63	17.08±0.61
	PE	19.96±1.21	20.33±3.57
$C_{18=1}$	PC	24.68±1.13	20.31±2.83
	PE	17.75±0.65	13.04±3.10[b]
$C_{18=2}$	PC	14.36±1.39	15.72±1.98
	PE	23.27±1.48	18.68±3.72
$C_{20=4}$	PC	2.52±0.23	3.02±0.54
	PE	11.25±0.24	19.71±0.33[a]
$C_{22=6}$	PC	1.80±0.44	1.57±0.33
	PE	3.12±1.57	5.08±1.32

Only the major acids are given. Each value represents the mean ±S.D.
a) $p<0.01$; *b*) $p<0.05$. The column and injection port temperatures were 185° and 205°, respectively.

Effect of OK-432 on AH 41 C Cells

1) **Total Number, Components and Enzyme Activities of AH 41 C Cells**——The number of the cells, obtained on day 11, was only slightly decreased by the two days-treatment of OK-432, the control group; 2.01±0.19 and OK-432 group; 1.87±0.14 ($\times 10^8$ cells/ml). After dosing OK-432 for 2 days, a clear decrease in calcium and potassium content of the tumor cells was shown as compared with the control cells, as shown in Table IV. However, other

Table IV. Effect of OK-432 on Cellular Components and Enyzme Activities of AH 41 C Cells

Component	Control	OK-432
Sialic acid	10.04 ±1.89	12.80 ±1.35
Amino sugar	11.31 ±2.14	10.76 ±1.10
Phospholipid phosphorus	0.160±0.019	0.131±0.017
Potassium	17.52 ±0.59	8.84 ±1.64[a]
Calcium	0.20 ±0.01	0.16 ±0.02[a]
Hexokinase	16.90 ±1.78	17.50 ±1.02
ATPase	3.60 ±0.47	3.57 ±0.32

OK-432 (0.5 mg/rat/day) was administered from day 9 to 10 after inoculation of the tumor cells. Each value represents the mean ±S.D. The units are the same as expressed in Table I.
a) $p<0.01$.

parameters such as both enzyme activities, ANS-cell fluorescence intensity and TNBS binding to phosphatidylethanolamine were not significantly affected by administration of OK-432 for 2 days.

2) **Fatty Acid Composition of Neutral Lipids and Phospholipids of AH 41 C Cells**—— The fatty acid composition of phospholipids of the cells is shown in Table V. A partial decrease in stearic acid (10%) and a significant increase in arachidonic acid (33%) were found as compared with the control. This result suggests that the fatty acid composition of phospholipids is partially modified at the early stage after administration of OK-432. The percentage composition of fatty acids of neutral lipids was little or no change except palmitic acid of which content was decreased to 88% of the control.

Table V. Fatty Acid Composition of Phospholipids of AH 41 C Cells treated with OK-432

Fatty acid	Control (%)	OK-432 (%)
$C_{16=0}$	24.38±1.67	23.05±2.75
$C_{18=0}$	24.35±1.27	21.90±0.82[a]
$C_{18=1}$	14.89±1.28	15.28±0.85
$C_{18=2}$	16.91±1.37	16.76±1.72
$C_{20=4}$	6.63±0.30	8.82±1.00[b]
$C_{22=6}$	2.19±0.80	2.77±0.78

Only the major acids are given. Each value represents the mean ±S.D.
a) $p<0.05$; *b*) $p<0.01$.

3) **Fatty Acid Composition of Phosphatidylethanolamine and Phosphatidylcholine**—— The fatty acid composition of these phospholipids of AH 41 C cells is shown in Table VI. After dosing OK-432 for 2 days, a clear per cent decrease in steric acid and an increment in arachidonic acid were observed in phosphatidylethanolamine. Although linoleic acid in phosphatidaylcholine was only slightly increased, a large change in the fatty acid composition of this phospholipid was not shown.

Table VI. Fatty Acid Composition of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) of AH 41 C Cells Treated with OK-432

Fatty acid		Control (%)	OK-432 (%)
$C_{16=0}$	PC	17.58±1.33	17.51±1.06
	PE	10.64±1.15	7.88±1.69
$C_{18=0}$	PC	27.41±1.77	25.38±1.33
	PE	24.48±1.69	17.44±3.20[a]
$C_{18=1}$	PC	16.25±1.90	16.38±1.31
	PE	17.28±0.83	14.38±1.88
$C_{18=2}$	PC	15.26±0.62	17.81±0.56[b]
	PE	18.65±3.28	21.40±1.71
$C_{20=4}$	PC	8.56±1.28	8.84±0.93
	PE	13.18±0.42	15.42±1.05[a]
$C_{22=6}$	PC	5.19±2.48	3.92±1.72
	PE	4.22±0.63	5.26±1.68

Only the major acids are given. Each value represents the mean ±S.D.
a) $p<0.05$; *b*) $p<0.01$.

Effect of 5FU and CP on AH 130 Cells

The effect of 5FU and CP on some components and fatty acid composition of phospholipids in AH 130 cells was tested in comparison with that of OK-432. As a result, the content of sialic acid, potassium and calcium and other parameters as shown in Table VII were scarcely

changed by administration of CP. Additionally, the fatty acid composition of phospholipids was not subjected to a significant change by the treatment with 5FU and CP except that the content of arachidonic acid in phospholipids was slightly enhanced after administration of 5FU (the control; 6.00±0.65, 5FU; 7.69±0.34, $p<0.01$). These results indicate that there was not a significant alteration of the cell membrane after dosing 5FU and CP.

Table VII. Effect of Cyclophosphamide on Cellular Components and ANS-fluorescence Intensity of AH 130 Cells

Component	Control	Cyclophosphamide
Sialic acid	7.02±0.28	7.41±0.16
Amino sugar	9.90±0.69	10.42±0.82
Potassium	27.32±0.79	27.69±2.52
Calcium	0.16±0.02	0.18±0.02
ANS-fluorescence intensity	1.16±0.10	1.28±0.04

Each value represents the mean ±S.D. and the units are the same as expressed in Tables I and II.

Discussion

OK-432, which is produced from streptococcal cells,[4a] was found to be effective against various kinds of ascites and solid forms of tumors,[4] by both a direct cytocidal action[19] and host-mediated antitumor activity.[4a,20] However, the biochemical mechanism of its action at the early stage remains unclear. Therefore, attention has turned to studies designed to elucidate the mechanism of cytolytic activity of OK-432.

In the present study, the injection of OK-432 to AH 130 cell-bearing rats for 4 successive days caused the increased leakage of potassium and calcium from the cells (Table I) and the enhanced binding of TNBS to phosphatidylethanolamine (Table II). These alterations produced with OK-432 are probably due to the increased permeability of the membrane. It is suggested that the rate of the reaction of phosphatidylethanolamine with TNBS is used as an indicator for such an alteration of lipid-protein interactions and the increase in accessibility of the phospholipid resulted from changes of the arrangement of lipid-protein interactions normally stabilized by glycolytic metabolism.[21] Thus, the profound perturbation of lipid-protein interactions and consequently an increased disturbance of the physical state of the tumor cell membrane appear to be produced by administration of OK-432. The enhanced perturbation probably induces the leakage of intracellular components vital to the survival of the cells, thereby inhibiting the cell growth. The result that ANS-cell fluorescence intensity was little affected but TNBS binding was enhanced by the treatment of OK-432 suggests that the overall organization of the membrane lipids and proteins was scarcely changed, whereas the arrangement of some phospholipids was subjected to partial alterations with the agent.

The treatment with the agent is considered to give an important influence on the lipid metabolism of the cells. An increased proportion of polyenoic fatty acids, *e.g.* arachidonic acid, and a decrease in palmitic and stearic acids in phospholipids (Fig. 4) appear to produce the changes in the physical state and enhancement of the fluidity of the cell membrane. The changes might be associated with the increased permeability of the cell membrane. This suggestion has received support from the findings that alterations in the saturation of fatty

19) H.F. Havas, A.J. Donnelly, and A.V. Porreca, *Cancer Res.*, **23**, 700 (1963); S. Koshimura, R. Shimizu, Y. Bando, R. Hirata, and S. Shoin, *Jpn. J. Microbiol.*, **4**, 19 (1960).
20) I. Kimura, T. Onoshi, J. Takano, H. Osawa, S. Yasuhara, T. Watanabe, M. Sugiyama, and K. Hiraki, *Zikken Igaku Zasshi*, **50**, 2965 (1973).
21) C.W.M. Haest and B. Deuticke, *Biochim. Biophys. Acta*, **401**, 468 (1975).

acid supplements affected the membrane transport properties and enzyme activity of *Escherichia coli* auxotrophs,[22] and that the fluidity and permeability of the cell membrane are strongly affected by the fatty acid composition of the phospholipids.[23] It is of much interesting that the fatty acid composition of phosphatidylethanolamine in the cells treated with OK-432 was significantly altered in comparison with that of the control tumor cells, while the change in phosphatidylcholine was not induced (Table III). This suggests that the alterations mediated by OK-432 are specific for phosphatidylethanolamine and probably for some phospholipid species.

Inbar *et al.* indicated that transformed fibroblasts had a more rigid lipid layer as compared with normal fibroblasts[24] and this was based on the composition of the cell lipid components and primarily induced by differences in the molar ratio between cholesterol and phospholipids. The increase in the ratio was suggested to induce a decrease in the degree of membrane fluidity.[25] The effect exerted by increased content of cholesterol in the cells treated with OK-432, however, will be negated by the significantly increased ratio of unsaturated fatty acids and the decrease in saturated fatty acids. Therefore, the fluidity of the cell membrane might be enhanced by the treatment of OK-432.

Knox *et al.* showed that there was good correlation between the growth rate of transplantable tumor cells and the hexokinase activity.[26] The decreased hexokinase activity after administration of OK-432 for 4 days (Table I) thus seems probable to cause the partial inhibition of glycolysis in the tumor cells and consequently provides inhibition of growth and proliferation of tumor cells. The decreased activity may be due to the leakage of the enzyme from the cells, since the cells incubated with OK-432 (10^7 cells/0.5 mg/ml) in Eagle MEM containing 10% fetal bovine serum for 3 hr at 37° lost 21% of the hexokinase activity and part of the activity was found in the outer medium. ATPase activity of AH 130 and AH 41 C cells was not affected by the administration of OK-432 for 4 or 2 days, respectively, indicating that the cytolytic activity mediated with OK-432 at the early stage may be not due to regulation of ATPase.

OK-432 was found to be much more effective against AH 41 C cells than against AH 130 cells.[4a] This is in good agreement with our data of *in vivo* cytolytic activity of OK-432 (Fig. 1). One reason why AH 41 C cell-bearing rat was treated with OK-432 for only 2 days was to allow examination of the direct *in vivo* cytocidal activity, because an increase in the number of macrophages was observed at 3 days after injection of OK-432 (0.02 mg/mouse)[27] and the host-mediated antitumor activity may be strongly exerted at 4 days after its administration. As a result, a single daily dose of OK-432 for 2 days to tumor (AH 41 C cell)-bearing rats caused a loss of potassium and calcium, a clear per cent decrease in stearic acid and a significant increase in arachidonic acid. The alterations might also partially produce the changes in the membrane permeability and fluidity.

These alterations induced by administration of OK-432 as mentioned above might be specific for AH 130 and AH 41 C cells, since the cells treated with 5FU and CP did not show such a significant change as observed in OK-432 treatment (Table VII).

These experiments on the antitumor activity of OK-432 lead to the conclusion that administration of the agent to ascites tumor-bearing rats causes the enhancement of membrane

22) G. Wilson, S.P. Rose, and C.F. Fox, *Biochem. Biophys. Res. Commun.*, **38**, 617 (1970); P. Overath, H.U. Schairer, and W. Staffel, *Proc. Nat. Acad. Sci. U.S.A.*, **67**, 606 (1970).
23) A.D. Keith, B.J. Wisnieski, S. Henry, and J.C. Williams, "Lipids and Biomembranes of Eukaryotic Microorganisms," ed. by J.A. Erwin, Academic Press, New York, 1973, p. 259; G.A. Thompson Jr. and Y. Nozawa, *Biochim. Biophys. Acta*, **472**, 55 (1977).
24) M. Inbar, I. Yuli, and A. Raz, *Exp. Cell Res.*, **105**, 325 (1977).
25) M. Inbar and M. Shinitzky, *Proc. Nat. Acad. Sci. U.S.A.*, **71**, 4229 (1974); M. Shinitzky and M. Inbar, *Biochim. Biophys. Acta*, **433**, 133 (1976).
26) W.E. Knox, S.C. Jamder, and P.A. Davis, *Cancer Res.*, **30**, 2240 (1970).
27) H. Mashiba, M. Gojobori, and K. Matsunaga, *Gann*, **68**, 703 (1977).

permeability and fluidity at the early stage. This is partially due to an increased proportion of unsaturated fatty acids and a decreased content of saturated fatty acids in phospholipids. These alterations appear to induce leakage of essential components such as potassium, calcium and hexokinase from the cell, which at least partially resulting in the destruction of the cells and the inhibition of their growth. However, other processes not considered in this study may be involved in the antitumor activity of the agent. The enhanced permeability of the cell membrane with OK-432 suggests that in combined use of OK-432 and other antitumor agents, OK-432 may increase the instillation of other agents into tumor cells and consequently decrease their doses. Part of these effects may be due to cytostatic or cytotoxic factors released from macrophages activated with OK-432[28] besides a direct cytolytic action and not due to streptolysin S since the attenuated preparation was completely devoid of streptolysin S-producing ability.[4a]

Acknowledgement We wish to thank Chugai Seiyaku Co., Ltd. for providing OK-432 preparation.

28) W. Braun and M. Ishizuka, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 1114 (1971); J.J. Kramer and G.A. Granger, *Cell. Immunol.*, **3**, 88 (1972); R. Keller, *Cell. Immunol.*, **17**, 542 (1975); H.G. Opitz, D. Niethammer, H. Lemke, H.D. Flad, and R. Huget, *Cell. Immunol.*, **16**, 379 (1975); C.E. Piper and K.L. McIvor, *Cell. Immunol.*, **17**, 423 (1975).