Pilocarpine, a cholinomimetic drug like ACh, caused a selective strong contraction of the bronchus, and showed only a low potency on the bronchiole as compared with ACh, but the reason remains unexplained.

Nicotine produced only a slight contraction of all the portions of the respiratory tract. It is known that nerve plexus and ganglion cells are present under the mucosa of airways.[10] The number of ganglion cells under the mucosa is, however, smaller than that of ganglion cells situated outside the airway tissues. The low potency of nicotine in contracting the isolated airway smooth muscles may be due to a paucity of parasympathetic ganglion cells in the preparations.

It has been known that administration of *Ascaris suum* antigen by inhalation or *i.v.* injection to dogs with positive skin reaction provokes asthmatic responses such as tachypnea and increases in respiratory resistance and airway secretion which are reduced by antihistamines and atropine.[3,11] In addition, it has been recognized that histamine is released from the lung by inhalation of *Ascaris* antigen to dogs.[12] In the present Schultz-Dale test using airway smooth muscles of dogs sensitive to *Ascaris* antigen, the antigen introduced into the bath never produced contraction of their tissues. The result suggests that histamine released from mast cells may produce contraction, not by directly acting on smooth muscles, but rather by exciting vagal reflex.

10) D.F. Hawkins and W.D.M. Paton, *J. Physiol.*, **144**, 193 (1958).
11) *a*) W.M. Gold, G.-F. Kessler, and D.Y.C. Yu, *J. Appl. Physiol.*, **33**, 719 (1972); *b*) D.Y.C. Yu, S.P. Galant, and W.M. Gold, *J. Appl. Physiol.*, **32**, 823 (1972).
12) A. Chiesa, D. Dain, G.L. Meyers, G.-F. Kessler, and W.M. Gold, *Amer. Rev. Resp. Dis.*, **111**, 148 (1975).

[Chem. Pharm. Bull. 26(1) 321—324 (1978)]

UDC 547.458.04.09 : 615.277.011.5.076.9

Growth Inhibitory Activity of Synthetic Mannan Derivative against Tumor Cells *in Vitro*[1]

Tatsuji Matsumoto, Takeshi Mikami, Yuichi Sato, Masuko Suzuki, and Shigeo Suzuki

Tohoku College of Pharmacy[2]

(Received September 27, 1977)

A synthetic mannan derivative, stearoylmannan phosphate, inhibited the growth of sarcoma-180 C and HeLa-S_3 cells *in vitro*. Incorporation of ^{3}H-thymidine by HeLa-S_3 cells was inhibited by this compound and in synchronous culture, this compound inhibited the growth of HeLa-S_3 cells only when added immediately after removal of thymidine.

Keywords——yeast mannan; stearoylmannan phosphate; antitumor polysaccharide; antitumor activity; sarcoma-180 C; HeLa-S_3; liquid scintillation counter; synchronous culture; mannan derivative

We have already reported that mannan and glucan extracted from baker's yeast showed an antitumor activity against the solid tumor of sarcoma-180 in mice,[3] and that the polysac-

1) *a*) This paper constitutes Part IX of a series of "Antitumor Activity of Polysaccharides." *b*) Part VIII: M. Suzuki, T. Mikami, M. Kadowaki, T. Matsumoto, and S. Suzuki, *Cancer Res.*, **37**, 3448 (1977); *c*) This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.
2) Location: *Komatsushima 4-4-1, Sendai, 983 Japan.*
3) S. Suzuki, H. Hatsukaiwa, H. Sunayama, M. Uchiyama, F. Fukuoka, M. Nakanishi, and S. Akiya, *Gann*, **60**, 65 (1969).

charides regressed the solid tumor by their adjuvant action.[4] It has been found that most of the antitumor polysaccharides show their effect only against solid tumors.[5] We have attempted to introduce a substituent group having an antitumor activity by itself into antitumor polysaccharides in order to develop polysaccharide derivatives which have both the host-mediated and direct action on cancer cells.[6] Stearoylmannan phosphate, obtained by introduction of fatty acid and phosphate groups into yeast mannan, showed a strong antitumor activity against Ehrlich ascites tumor when used in conjunction with ineffective dose of mitomycin-C,[7] although a combined use of mannan and mitomycin-C was found to be ineffective. This result indicates that stearoylmannan phosphate has an activity different from that of mannan.

The present paper is an attempt to elucidate the mechanism of stearoylmannan phosphate on tumor cells *in vitro*.

Materials and Methods

Extraction and Purification of Mannan from Baker's Yeast——These procedures followed the method of Suzuki and Sunayama,[8] using fresh baker's yeast (Oriental Yeast Ind., Tokyo).

Mannan Derivative——Synthesis and Purification of Stearoylmannan Phosphate: Stearoylmannan phosphate was synthesized and fractionated as reported previously.[6]

Cells and Culture Conditions——HeLa-S_3 cells were kindly provided by Dr. I. Yamane, Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai, and sarcoma-180 C by Dr. K. Koyama of the National Cancer Center, Tokyo. The cells were cultured in Eagle's minimum essential medium (MEM) (GIBCO, New York, U.S.A.) added with 15% bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were cultured in test tubes and, 24 hr after start of the culture, medium was replaced with 1 ml of a new medium containing 1 mg/ml of stearoylmannan phosphate. The cells were detached from the test tubes,[9] collected, and stained with safranin solution[10] to count the number of dead and viable cells using a hemocytometer. Six test tubes were used for each time period and the experiment was repeated three times.

Incorporation of ^{3}H-thymidine into HeLa-S_3 Cells——HeLa-S_3 cells (1×10^5 cells/tube) were cultured as above, 1 mg/ml of stearoylmannan phosphate solution was added by the method of medium exchange 24 hr later, and 1 μCi of ^{3}H-thymidine was added 1 hr later. This mixture cultured at 37° for 24 hr and incorporation of ^{3}H–thymidine was measured with a liquid scintillation counter according to the method of Takiguchi *et al.*[11] The culture was carried out in 20 test tubes for each group, and the experiment was repeated twice. The radioactivity was determined in an automatic liquid scintillation spectrometer (Beckman LS-150).

Synchronous Culture of HeLa-S_3 Cells——About 1×10^5 HeLa-S_3 cells were cultured in a test tube, the medium was replaced with the same medium containing 2 mM of thymidine[12] after 16 hr, and further cultured for 24 hr. The cells were collected, washed three times with 3 ml each of Hanks' balanced salt solution to remove thymidine, and returned to the normal medium. One milliliter of a solution containing 1 mg/ml of stearoylmannan phosphate was added to the test tubes immediately after removal of thymidine or immediately before the cells went into mitosis. After incubation, the cells were then treated with 0.1 M citric acid at 37° for 1 hr, stained with 0.1% Gentian Violet, and the number of nuclei was counted with a hemocytometer.[13] Ten test tubes were used for each time period and experiment was repeated twice.

4) *a*) S. Suzuki, M. Suzuki, T. Matsumoto, and Y. Okawa, *Gann*, **62**, 343 (1971); *b*) M. Suzuki, F. Chaki, and S. Suzuki, *Gann*, **62**, 553 (1971).
5) *a*) N. Komatsu, S. Okubo, S. Kikumoto, K. Kimura, G. Saito, and S. Sakai, *Gann*, **60**, 137 (1969); *b*) G. Chihara, J. Hamuro, Y. Maeda, Y. Arai, and F. Fukuoka, *Cancer Res.*, **30**, 2776 (1970).
6) M. Suzuki, T. Matsumoto, T. Mikami, and S. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **24**, 1100 (1976).
7) M. Suzuki, T. Matsumoto, T. Mikami, and S. Suzuki, *Gann*, **67**, 607 (1976).
8) S. Suzuki, and H. Sunayama, *Tanpakushitsu Kakusan Kôso*, **19**, 1094 (1974).
9) N. Ishida, "Experimental Code for Microbiology and Serology," (in Japanese) ed. by N. Ishida, H. Zenyoji, and C. Matsuhashi, Nanzando, Tokyo, 1966, p. 201.
10) A. Oda, *Jpn. J. Expt. Med.*, **29**, 79 (1959).
11) T. Takiguchi, T. Hisada, and S. Kishida, "Experiment in Immunology," (in Japanese) Japanese Society of Immunology, 1970, p. 315.
12) N. Xeros, *Nature* (London), **194**, 682 (1962).
13) T. Kunimoto, M. Hori, and H. Umezawa, *J. Antibiot., Ser. A*, **20**, 277 (1967).

Results

Effect of Stearoylmannan Phosphate on Tumor Cells *in Vitro*

Effect of stearoylmannan phosphate on cultured cells of sarcoma-180C was tested by the addition of 1 mg/ml of stearoylmannan phosphate to the medium and number of viable cells was examined 1, 24, and 48 hr later. At 1 hr after the addition, the number was no different from that of the untreated control group but, after 24 and 48 hr, cell multiplication decreased, and approximately the same effect as that of the positive control, 1 μg/ml of mitomycin-C, was found. The number of dead cells did not increase at any of the periods after addition of stearoylmannan phosphate, indicating that this fraction had no cytocidal effect and only inhibited cell multiplication. However, the mannan did not decrease cell multiplication after 1, 24, and 48 hr (Fig. 1). As shown in Fig. 2, similar examination with HeLa-S_3 cells showed that, at 72 hr, multiplication of HeLa-S_3 cells decreased at the dose of 1 mg/ml, as in sarcoma-180C cells. On the other hand, at the dose of 1 and 100 μg/ml, stearoylmannan phosphate had no effect on the multiplication of HeLa-S_3 cells after 24 and 48 hr of incubation but 29% growth inhibition was observed after 72 hr at 100 μg/ml, while the mannan had no effect at any of the periods.

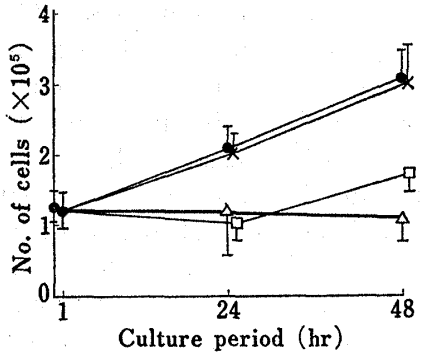

Fig. 1. Growth Curves of Sarcoma-180C Cells in Medium with Stearoylmannan Phosphate

—●—, control.
—△—, stearoylmannan phosphate, 1000 μg/ml.
—×—, mannan, 1000 μg/ml.
—□—, mitomycin-C, 1 μg/ml.

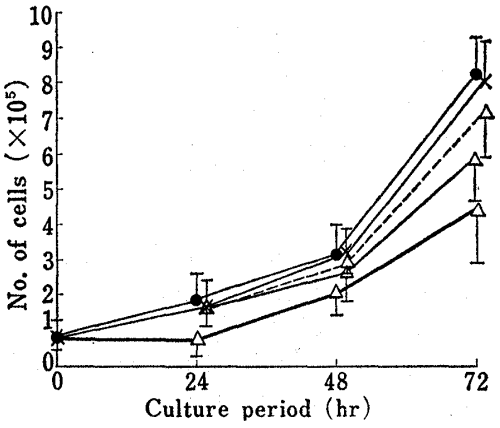

Fig. 2. Growth Curves of HeLa-S_3 Cells with Stearoylmannan Phosphate

—●—, control.
—△—, stearoylmannan phosphate, 1000 μg/ml.
—△—, stearoylmannan phosphate, 100 μg/ml
---△---, stearoylmannan phosphate, 1 μg/ml.
—×—, mannan, 1000 μg/ml.

Effect of Stearoylmannan Phosphate on the Incorporation of ^{3}H-thymidine into HeLa-S_3 Cells

Incorporation of radioactivity into HeLa-S_3 cells after addition of 1 mg/ml of stearoylmannan phosphate was 181 465±13 473 dpm on the average, indicating about 32% inhibition compared to that of untreated cells, and the difference was statistically significant ($p<0.01$). Incorporation of ^{3}H-thymidine by HeLa-S_3 cells given 1 mg/ml of mannan was no different from that of untreated cells (Table I).

Table I. Uptake of ^{3}H-thymidine by HeLa-S_3 Cells

Sample	Concentration (mg/ml)	Radioactivity (dpm/min)	Inhibition ratio (%)	*P* value[a]
Control		268 373± 9 824		
Mannan	1.0	246 126±18 761	8	
Stearoylmannan phosphate	1.0	181 465±13 473	32	<0.01

a) *Versus* the control group.

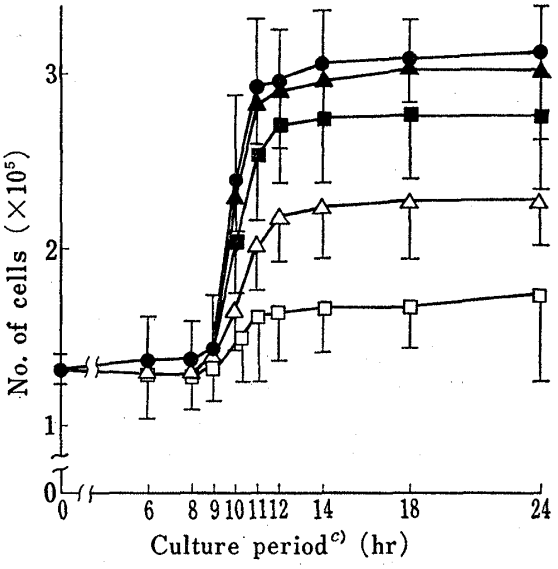

Fig. 3. Effect of Stearoylmannan Phosphate on HeLa-S_3 Cell Multiplication

—●—, control.
—▲—, stearoylmannan phosphate, 1000 μg/ml.[a]
—△—, stearoylmannan phosphate, 1000 μg/ml.[b]
—■—, mitomycin-C, 1 μg/ml.[a]
—□—, mitomycin-C, 1 μg/ml.[b]
a) Added 9 hr after removal of thymidine.
b) Added just after removal of thymidine.
c) Culture period indicates the time after removal of excess thymidine.

Action Site of Stearoylmannan Phosphate in Synchronized HeLa-S_3 Cells

HeLa-S_3 cells were synchronized with thymidine and the site of action of stearoylmannan phosphate was examined by the time of its addition. Effect of stearoylmannan phosphate on the growth of HeLa-S_3 cells was examined by its addition immediately after removal of thymidine or immediately before the start of mitosis. Decrease in the multiplication of mitotic cells was seen only when stearoylmannan phosphate was added immediately after removal of thymidine and not when added immediately before the start of mitosis, *i.e.*, 9 hr after removal of thymidine (Fig. 3).

Discussion

Yeast mannan has an antitumor activity against solid form of sarcoma-180, but no activity against ascites form of the tumor cells.[3] On the other hand, stearoylmannan phosphate, a modified mannan derivative, showed an antitumor effect when used with an ineffective dose of mitomycin-C against ascites form of Ehrlich carcinoma, but did not show an antitumor effect against solid form of tumor.[7] The mannan also had the effect of increasing antibody production and cellular response,[4] while stearoylmannan phosphate did not show any activity.[14] These facts signify that the action site of mannan and synthesized stearoylmannan phosphate on tumor cells would be different. Therefore, we examined the action of these two substances on tumor cells *in vitro*, and found that stearoylmannan phosphate inhibited the growth of tumor cells, sarcoma-180C and HeLa-S_3 cells, *in vitro*, but the mannan had no effect on tumor cells (Fig. 1 and 2). Stearoylmannan phosphate inhibited uptake of ^{3}H-thymidine (Table I) and inhibited the growth of HeLa-S_3 cells at the start of DNA synthesis as did mitomycin-C (Fig. 3). However, elucidation of the site of action of this growth inhibitory effect requires further detailed examinations.

We have reported the antitumor activity of palmitoyldextran phosphate,[15] and this compound had an antitumor activity against both solid and ascites form of tumor *in vivo*. However, palmitoyldextran phosphate had no effect on tumor growth *in vitro*, indicating that the mechanism of antitumor activity was different from that of stearoylmannan phosphate.[1b] Stearoylmannan phosphate and palmitoyldextran phosphate are different from these polysaccharides, *i.e.*, mannan consists of a highly branched polymer of mannose,[8] and dextran consists of a linear polymer of glucose.[16] It is interesting that both polysaccharide derivatives have different antitumor and immunological activities.

Acknowledgment Authors thank Dr. N. Kumano, the Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, for the measurement of radioactivity.

14) T. Matsumoto, M. Suzuki, and S. Suzuki, Proc. Jpn. Cancer Assoc., 34th Annu. Meet., 82 (1975).
15) M. Suzuki, T. Mikami, T. Matsumoto, and S. Suzuki, *Carbohyd. Res.*, 53, 223 (1977).
16) A. Jenes, W.C. Hanes, C.A. Wilham, J.C. Rankin, E.H. Melvin, M.J. Clusky, B.E. Fisher, H.M. Tsuchiya, and C.E. Rist, *J. Am. Chem. Soc.*, 76, 5401 (1954).