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Stimulation of Macrophage by Polyanions and Its Conjugated Proteins and Effect on Cell Membrane[1] (42218)

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Abstract. Lipophilic anionic copolymer (styrene-maleic acid; SMA) conjugates of albumin and antitumor protein neocarzinostatin (NCS) (smancs) were found to stimulate the release of H_2O_2 and O_2^- from the peritoneal macrophages obtained from mice which had been pretreated with the heat-killed preparation of *Streptococcus pyogenes* (OK-432) *in vivo*. Some alkyl esters of SMA exhibited effects similar to protein–polymer conjugates. Among them, butyl-SMA was the most effective followed by ethyl-SMA, whereas hydrolyzed SMA showed no effect. This activity was dose-dependent but exhibited a bell-shape profile. These results suggest that the aliphatic ester residue in SMA as well as the main chain of the copolymer may be important for the activation of macrophages. A strong antitumor effect of smancs reported elsewhere may be attributed partly to the activation of macrophages in addition to the direct damage to the cellular DNA by the NCS component. A preliminary investigation of the subcellular mechanism of macrophage activation was carried out in view of membrane fluidity by the fluorescence polarization method. The results showed that the apparent decrease in the cell membrane fluidity and the degree of macrophage activation paralleled the same dose range and at similar time courses. This indicated the interaction of SMA component and macrophage cell membrane. © 1986 Society for Experimental Biology and Medicine.

A chemical conjugate of a synthetic copolymer of styrene and maleic acid (SMA)[2] with a proteinaceous antitumor agent, neocarzinostatin (NCS), was initially prepared and was designated as smancs (1–3). Smancs has been shown to exhibit a pronounced antitumor effect against animal and human tumors *in vivo* (2, 4–8). The marked antitumor activity may be attributed to its effects on DNA (5, 9). However, the effect of smancs on the immunological system has remained to be clarified.

Macrophages are known to release H_2O_2 and O_2^- into the medium during phagocytosis (10) or in response to various membrane activators such as phorbol myristate acetate (11) and concanavalin A (12). Meanwhile, considerable evidence seems to indicate that H_2O_2 and O_2^- play an important role in antimicrobial and antitumor activity in host animals (13–15). The present study was undertaken to examine such stimulation of macrophages by various SMA copolymers and their protein conjugates. Furthermore, the subcellular mechanism of action was investigated for the effects on the membrane fluidity using the fluorescence polarization method.

Materials and Methods. *Mice.* ddy male mice, 7 to 10 weeks old, were obtained from Kyudo Laboratories Company, Ltd. (Tosu, Japan).

Reagents. Smancs was prepared in this laboratory as described previously (1–3). SMABSA was prepared similarly although about 50% of the amino groups had no conjugation reaction. It was purified with Sephadex G-75 following dialysis in 50 m*M* ammonium bicarbonate. NCS was obtained from Kayaku Company, Ltd., Tokyo. SMA and its various aliphatic esters were prepared by Kuraray Company Ltd., Osaka, for us. Conca-

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[2] Abbreviations used: NCS, neocarzinostatin; SMA, a copolymer of styrene and maleic acid of which half of the carboxyl group is *n*-butylated as ester unless otherwise specified, and its average molecular weight ranged from 2000 to 2500; SMABSA, SMA-conjugated bovine serum albumin; PMA, phorbol myristate acetate; ConA, concanavalin A; WGA, wheat germ agglutinin; SOD, superoxide dismutase; KRP, Krebs–Ringer phosphate buffer; HBSS, Hanks' balanced salt solution; DMSO, dimethyl sulfoxide; PEC, peritoneal exudate cells; DPH, 1,6-diphenyl-1,3,5-hexatriene; RBC, red blood cells; FCS, fetal calf serum; FP value, fluorescence polarization value in arbitrary units.

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navalin A (ConA), phorbol myristate acetate (PMA), wheat germ agglutinin (WGA), scopoletin, superoxide dismutase (SOD), and cytochrome *c* (Type IV) were purchased from Sigma Chemical Company, St. Louis, Missouri. PMA was dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 1 mg/ml as a stock solution and the same blank DMSO solution was used as a control. Other agents were dissolved in Krebs–Ringer phosphate buffer (KRP). OK-432 (16), a heat-treated lyophilized preparation of a low virulence strain of *S. pyogenes* with penicillin, was kindly provided by Chugai Pharmaceutical Company, Ltd., Tokyo. One KE unit (Klinishe Einheit) of OK-432 contains 0.1 mg of the dried cocci (10^7–10^8), which was used after suspending in Hanks' solution. 1,6-Diphenyl-1,3,5-hexatriene (DPH; Aldrich Chemical Co.) and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan, and Percoll from Pharmacia Fine Chemicals Co., London. Other reagents were of analytical grade.

Preparation of peritoneal macrophages. Male ddy mice were injected intraperitoneally with 1 KE of OK-432 in 0.1 ml of Hanks' balanced salt solution (HBSS) to elicit peritoneal macrophages. The resulting peritoneal exudate cells (PEC) were harvested 3 days after injection by peritoneal lavage using 10 ml of cold HBSS containing 10 U heparin/ml yielding 1–2 $\times$ 10^7 cells per mouse. After centrifugation at 200*g*, the cell pellet was resuspended in 2 ml of 0.2% saline for 30–60 sec to lyse erythrocytes. Isotonicity was restored with 1.6% saline followed by the addition of 6 ml HBSS, and centrifugation was repeated. The cells were then resuspended in cold RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), of which 0.5-ml aliquots were plated in 16-mm wells (Falcon 24-well plates) at a density of 5×10^5/well, and incubated for 3 hr at 37°C in the incubator (5% CO_2 and 95% air). Then the medium was removed and the adherent cells were washed vigorously three times with HBSS to remove nonadherent cells. In some experiments, peritoneal cells were plated on 9.5 $\times$ 9.5-mm flying coverslips in 35-mm plastic culture dishes with 2 ml of medium for 3 hr. The coverslips were washed vigorously three times with HBSS to remove nonadherent cells and added directly to cuvettes containing KRP for H_2O_2 or O_2^- assay. More than 95% of these adherent cells exhibited the characteristics of macrophages.

For the fluorescence polarization measurement, peritoneal macrophages were similarly purified from mice with OK-432. The peritoneal exudate cells were washed three times with RPMI 1640 medium and resuspended in the same medium. Of the cell suspension 0.5 ml was layered on the top of 9 ml of 58% Percoll in RPMI-1640 medium, and centrifugated at 400*g* for 30 min at 4°C. The nonsedimented cells containing more than 90% of macrophages were collected and similarly washed three times with KRP, followed by resuspension in KRP to a concentration of 2 $\times$ 10^6 cells/ml, and used for labeling with DPH.

Red blood cells. Human red blood cells (RBCs) were obtained from heparinized blood of healthy donors (Type O). The blood was centrifuged at 300*g* for 5 min at room temperature and the plasma and buffy coat were removed carefully by aspiration. The precipitated cells were then washed three times with ice-cold PBS and the RBC suspension at a concentration of 1% (v/v) was prepared by adding an appropriate volume of PBS to packed erythrocytes.

Assay for H_2O_2 release. Production of H_2O_2 by macrophages was quantitated by the scopoletin method as described (11), with some modifications, based on the horseradish-peroxidase-dependent oxidation of scopoletin by H_2O_2 which was carried out in a 24-well plastic plate (Falcon). For the time-course assay, macrophages adherent on the coverslip were placed on the bottom of a cuvette containing 0.5 ml of KRP, 1 nmol of scopoletin, and 15 U of horseradish peroxidase in a temperature-controlled compartment (37°C) in the spectrophotometer. The decrease of fluorescence was recorded continuously and was correlated with the concentration of H_2O_2 by use of a standard curve of diluted H_2O_2 in cell free suspensions of scopoletin and horseradish peroxidase.

Assay for O_2^- release. Release of superoxide anion from macrophages was determined on the basis of decrease in the amount of ferricytochrome *c* by superoxide anions (17). To confirm the involvement of superoxide anion in the reduction of cytochrome *c*, 50 μg/ml

SOD was added to assay mixtures, by which the increase in absorption at 550 nm is quenched by SOD. The H_2O_2 and O_2^- releases were expressed as nanomoles per minute per milligram macrophage protein. After removal of the supernatant for H_2O_2 or O_2^- assay, adherent macrophages were solubilized by incubation in 0.5 *M* NaOH overnight at room temperature and the protein content was quantified (18). For assay of H_2O_2 and O_2^- triplicate experiments were made, and each assay value varied less than 5%.

Assay for lysozyme activity. Lysozyme activity was measured by the fluorescence polarization (FP-) method described by Maeda (19) using fluorescein-labeled peptidoglycan. Briefly, 0.4 ml of sample was added to 1.6 ml of PBS containing 10 μg of FITC-labeled substrate in cuvettes. After incubation for 10 min at 30°C, the FP value was measured with the fluorescence spectropolarimeter. From the relationship between the change in FP value and the amount of standard lysozyme, the lysozyme content in a sample was quantified. The total lysozyme in macrophages was extracted with *n*-butanol (20) and measured as described above.

DPH labeling and fluorescence polarization measurements. For monitoring the membrane fluidity we have used 1,6-diphenyl-1,3,5-hexatriene as a probe of the fluorescence, which is an efficient fluorescence polarization probe for lipid compartment (21). A solution of 2 m*M* DPH in tetrahydrofuran was prepared freshly for each experiment, 0.1 ml of which was diluted 1000 times in KRP while being stirred vigorously. The obtained aqueous solution of 2 μ*M* DPH is clear and practically free of fluorescence. Cell suspensions (2×10^6 cells/ml) were labeled with an equal volume of the DPH solution for 30 min at 25°C. The interaction of DPH with the cells is followed by a steep increase in fluorescence intensity. Upon examination under a fluorescence microscope a strong fluorescence around the membrane and some microsomal compartments was observed. The unincorporated DPH was removed by three washes with KRP and the cells were resuspended in KRP to a concentration of 1×10^6 cells/ml.

The FP value was measured at 25 ± 0.05°C with the instrument described above (22). FP values were obtained automatically using excitation of DPH through a three-cavity filter at 366 nm and emission spectra at 430 nm using an aqueous solution of 1 *M* $NaNO_2$ as a filter eliminating the wavelength below 390 nm.

Measurement of hemolysis. The hemolytic activity of SMA derivatives was measured in conjunction with membrane perturbation using human RBCs as follows. The series of twofold dilutions of each sample were prepared in wells of microtiter plate (96 U shape), using PBS as the diluent in a final volume of 0.1 ml. The reaction was started by adding 0.1 ml of 1% RBC suspension to each well. The plate was shaken to disperse the cells evenly and then allowed to stand for 1 hr at 37°C, until they settled. RBCs in the bottom of the well were observed and the minimum concentration required to elicit hemolysis was determined.

Results. *Effect of various membrane-perturbing agents and various SMA derivatives on H_2O_2 release from macrophages.* As shown in Fig. 1A, H_2O_2 release was demonstrated clearly when 100 μg/ml of smancs was added to the peritoneal macrophages from OK-432-treated mice. The rate of H_2O_2 release remained constant over a 12-min period of observation. At the concentrations indicated, smancs exhibited more potent activity than ConA or WGA, although that of PMA was found to be the most potent effector with a latency of a few minutes.

To investigate the effect of smancs on macrophages in more detail, the dose–response curves for triggering of H_2O_2 release by smancs, butyl-SMA, NCS, and also SMABSA were examined (Fig. 1B). SMA and SMABSA induced the release of H_2O_2 similar to smancs and all showed a bell-shape dose response except NCS which failed to show any significant effect over the range of 1–10,000 μg/ml. These results indicated the important role of SMA component in smancs in stimulating the release of H_2O_2. However, H_2O_2 release from macrophages treated with these SMA derivatives was significantly inhibited by the addition of 1 mg/ml BSA. Among these agents SMABSA exhibited the highest stimulatory activity. In Table I arbitrary optimal concentrations of these stimulants and the molecular size are compared.

Effect of various SMA derivatives and NCS

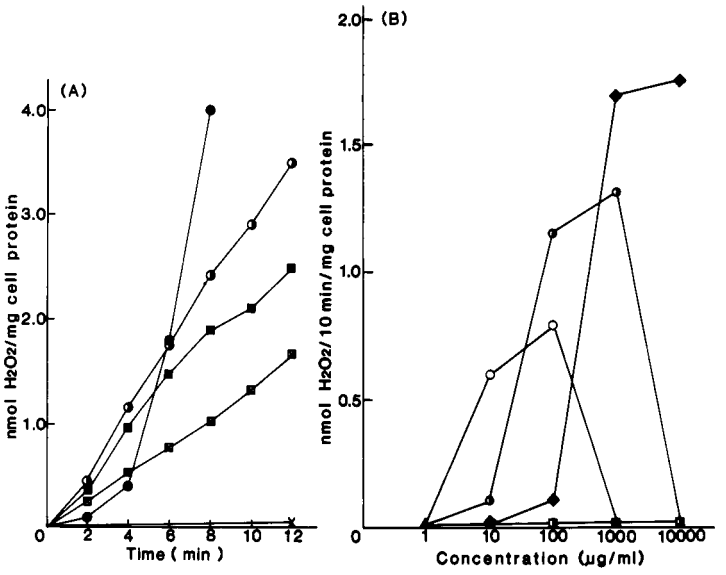

FIG. 1. H_2O_2 release from macrophages after the addition of various stimulants. (A) Time course. After the addition of 0.01 µg/ml PMA (●), 10 µg/ml WGA (■), 100 µg/ml ConA (⊠), or 100 µg/ml smancs (◑) to macrophages, the reduction of fluorescence intensity of scopoletin was recorded at 37°C for the indicated time. (×) The control; no stimulant. Results are expressed in terms of the adherent cell protein on matched coverslips. (B) Dose-response relationship. The resultant secretion of H_2O_2 from macrophages in response to various doses of smancs (◑), SMABSA (◆), SMA (○), or NCS (◨) was then quantified as in (A). The data are plotted as nanomoles of H_2O_2 secreted in 10 min per milligram of macrophages protein.

on O_2^- release. In polymorphonuclear leukocytes, H_2O_2 appears to arise during the respiratory burst mainly from the dismutation of superoxide anion (O_2^-) (23), which was produced primarily by the action of NADPH-oxidase (24). This pathway was examined in macrophages after treatment with various SMA derivatives. When macrophages were exposed to SMA or smancs, O_2^- production increased linearly (Fig. 2A). In the presence of 50 µg/ml SOD, the O_2^- release from macrophages in response to SMA derivatives and PMA was almost completely quenched. The dose-response curves of various SMA derivatives to O_2^- release correlated well with those of H_2O_2 release. However, NCS again had no effect (Fig. 2B).

TABLE I. OPTIMAL CONCENTRATIONS OF VARIOUS STIMULANTS AND H_2O_2 RELEASE FROM MACROPHAGES

Stimulant	Mol wt	Optimal concentration (µM)	nmol H_2O_2/10 min/mg cell protein
SMA	2,500	50	2.01 ± 0.35[a]
Smancs	17,000	5.9	2.95 ± 0.21
SMABSA	100,000	10	3.95 ± 0.11
NCS	12,000	—	0
ConA	104,000	0.96	1.32 ± 0.22
WGA	36,000	0.28	2.10 ± 0.50
PMA	600	0.017	35.0 ± 5.50

[a] Mean ± SD of three experiments.

Role of alkyl groups in the ester of SMA polymers on macrophage activation. SMA used in above experiments was a butyl ester derivative in which 50 to 70% (mole/mole) of the maleic acid carboxyl group were half esterified with butyl alcohol. To investigate the role of the alkyl ester group in macrophage stimulation, we compared butyl-SMA with ethyl- and nonesterified carboxylate forms of SMA polymers similarly. Figures 3A and 3B show the dose-response effect of these SMA polymers on the release of H_2O_2 and O_2^-. The results indicated that butyl-SMA was the most effective but ethyl-SMA, and hydrolyzed SMA had very little effect. However, conjugation of ethyl-SMA as well as butyl-SMA resulted in considerable increase in the potency (Fig. 3C).

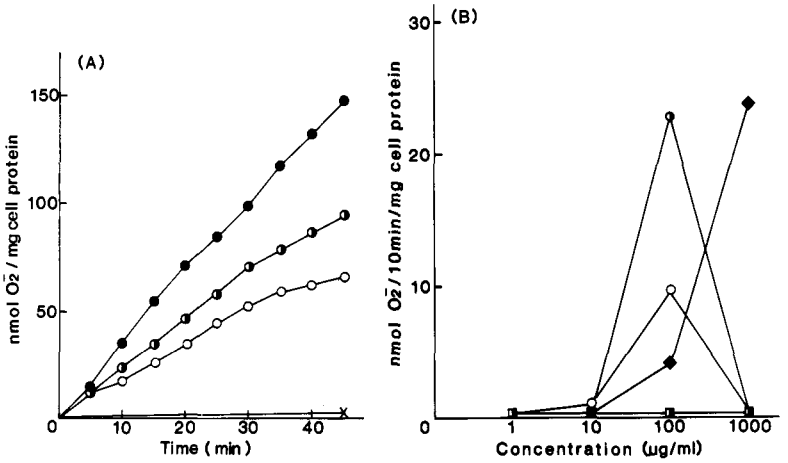

FIG. 2. O_2^- release from macrophages after the addition of PMA and SMA derivatives. (A) Time course. Macrophages were exposed to 0.01 μg/ml of PMA (●), 100 μg/ml of smancs (◑), or 10 μg/ml of SMA (○), and the resultant secretion of O_2^- was quantified at various times thereafter. (×) The control; no stimulant. Data are plotted as nanomoles of O_2^- secreted per milligram of macrophages protein against time. (B) Dose–response relationship. The secretion of O_2^- from adherent macrophages in response to various doses of smancs (◑), SMABSA (◆), SMA (○), or NCS (◨) was quantified as in (A). The data are plotted as nanomoles of O_2^- secreted in 10 min per milligram of macrophages protein.

Furthermore, we also found that lysozyme release was induced by butyl-SMA and ethyl-SMA depending on its concentration, but not by the carboxylate form of SMA (Fig. 3D).

Membrane fluidity of macrophages after treatment with various SMA derivatives. Some agents known to act on the cell membrane, such as surfactants (25) and phospholipase C (26), also stimulate the respiratory burst. Rossi *et al.* (27) have suggested that an alteration of the cell membrane chemically or by contact with bacteria triggers the activation of NADPH-oxidase. Thus, it is very likely that the effects of SMA derivatives are mediated by some modification of plasma membrane of macrophage. We investigated the physical effects of SMA derivatives on macrophage membrane by fluorescence polarization using DPH. As shown in Fig. 4, a marked increase of apparent FP values was observed when SMABSA, butyl-SMA, and ethyl-SMA were added to macrophages prelabeled with DPH, whereas the hydrolyzed free carboxylate form of SMA showed no effect. The increase in FP values was very rapid and dose dependent. Among these agents SMABSA was the most effective followed by butyl-SMA and ethyl-SMA, respectively. These results were very similar to those of previous data (Figs. 1–3) in both the profile of the time course and the concentration range of dose response. *n*-Butanol which was known to augment the membrane fluidity in a nonspecific manner was used as a control (28). It indeed reduced the apparent FP value. These experiments with DPH show that the addition of SMA derivatives to macrophages induces a very rapid change of apparent membrane fluidity. The effect of smancs and NCS on membrane fluidity could not be measured in this assay system due to intrinsic fluorescence of the NCS portion in the spectra range used in this experiment.

Hemolytic activity of various SMA derivatives. Exposure of washed RBCs to various SMA derivatives at 37°C for 1 hr resulted in hemolysis in the concentration-dependent manner of each derivative. Similar to the stimulation of H_2O_2 release described above, the hemolytic activities of these SMA derivatives were extensively inhibited by the addition of 1 mg/ml BSA. The minimum concentrations of SMA derivatives required to elicit hemolysis in the presence or absence of BSA (1 mg/ml) are summarized in Table II. Similar to macrophage activation, among three types of alkyl esters of SMA polymers, butyl-SMA was the most effective followed by ethyl-SMA,

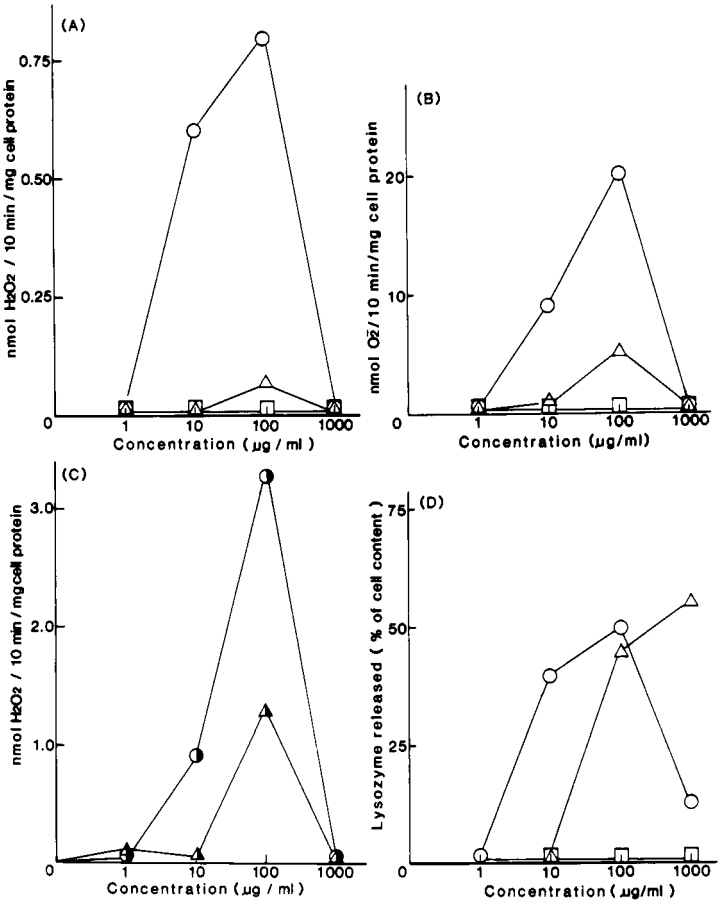

FIG. 3. Release of H_2O_2, O_2^- and lysozyme from macrophage after the addition of various SMA derivatives. (A) Dose response in H_2O_2 release. (B) Dose response in O_2^- release. The secretion of H_2O_2 or O_2^- from adherent macrophages in response to various doses of butyl-SMA (○), ethyl-SMA (△), or hydrolyzed SMA (□) was quantified. (C) Dose-response relationship of butyl- (◑) and ethyl-smancs (▲) in H_2O_2 release. Results are expressed as in Fig. 1B or 2B. (D) Dose-response relationship of various SMA polymers to lysozyme release. Adherent macrophages were incubated with various doses of butyl-SMA (○), ethyl-SMA (△), or hydrolyzed SMA (□) for 1 hr at 37°C and then lysozyme activity in supernatant fluid was assayed as described in the text. Lysozyme release is expressed as percentage of total cellular lysozyme content.

whereas the free carboxylate form of SMA had little effect. The hemolytic activities of smancs and SMABSA, both of which were conjugated with butyl-SMA, however, were much weaker than the butyl-SMA polymer.

Discussion. The results presented here (Figs. 1–3) show that alkyl ester derivatives of SMA and its protein conjugates including smancs induced both H_2O_2 and O_2^- release from mouse peritoneal macrophages which had been pretreated with OK-432 3 days earlier. Smancs exhibited more potent activity than ConA and WGA or its parental protein NCS (Fig. 1). The dose–response relationships of various agents for eliciting the release of H_2O_2 and O_2^- by treatment with smancs, SMA, NCS, and SMABSA were examined and it was found that SMA, smancs, and SMABSA showed bell-shape dose–response curves but NCS showed no effect (Figs. 1B, 2B). These results indicate that the SMA residues in both smancs and SMABSA might have an important role in the stimulation of macrophages. The types of alkyl esters of SMA copolymer showed critical importance in macrophage stimulation. Namely, the completely hydrolyzed free carboxylate

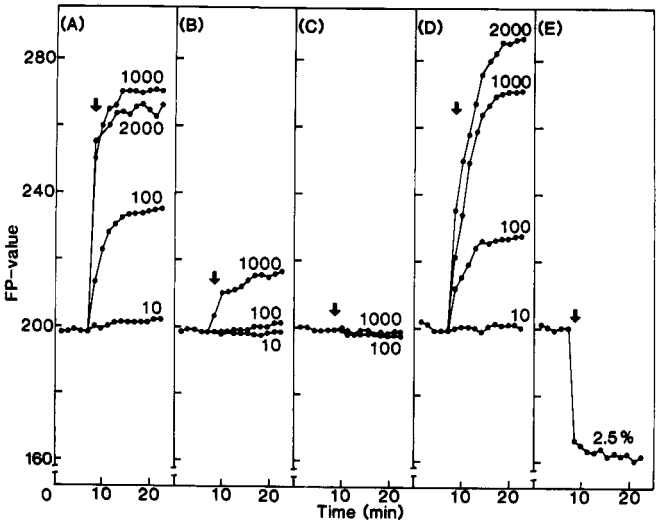

FIG. 4. Effect of various SMA derivatives and *n*-butanol on macrophage membrane fluidity. FP values of DPH bound to macrophages were measured at the indicated doses (μg/ml or %) at 25°C. (A) butyl-SMA; (B) ethyl-SMA; (C) hydrolyzed SMA; (D) SMABSA; (E) *n*-butanol, added at the arrows.

form had no effect, ethyl ester showed little effect, and butyl ester showed the highest effect as shown in Fig. 3. We have also found that lysozyme release was induced by butyl-SMA and ethyl-SMA, but not by the carboxylate form of SMA (Fig. 3D). Since the longer alkyl chains possess a higher hydrophobicity, the stimulatory effect of SMA on macrophages may be related to its hydrophobicity.

Kakinuma (29) reported that altered cellular integrity was required for stimulation of the respiratory burst. Perhaps SMA derivatives might induce some damage to cellular integrity. Thus, we preliminarily investigated the subcellular mechanisms which might be operating during this macrophage stimulation, as shown in Fig. 4, by fluorescence polarization. The results in Fig. 4 agree with those of Figs. 1–3, in both time-course profile and dose response. Therefore, the effect of SMA and its derivatives may involve alteration of membrane integrity for its stimulation of macrophage.

TABLE II. HEMOLYTIC ACTIVITY OF VARIOUS SMA DERIVATIVES IN THE PRESENCE OR ABSENCE OF 1 mg/ml OF BSA

Sample	Mol wt	Minimum conc for hemolysis (μM)	
		No BSA	With BSA (1 mg/ml)
Hydrolyzed SMA	2,500	500	2000
Ethyl-SMA	2,500	63	1000
Butyl-SMA	2,500	1.56	125
Smancs[a]	17,000	37 (74)[c]	590 (1180)[c]
SMABSA[b]	100,000	1.6 (16)[c]	50 (500)[c]

[a] Smancs contained 2 mol butyl-SMA polymers/mol NCS.

[b] SMANCS contained about 10 mol butyl-SMA/mol BSA.

[c] The values in parentheses are expressed as concentrations of butyl-SMA.

In a separate experiment we observed, in fact, that morphological changes in macrophages and erythrocytes were produced by SMA at the maximal dose (300 μg/ml) after prolonged incubation resulting in cell lysis (Table II). Furthermore, we found that the extent of erythrocyte lysis of SMA and SMA derivatives paralleled the data of macrophage stimulation (Table II).

The lytic effects of smancs and SMABSA on macrophages were milder than SMA alone. The magnitude of the lytic effect of these SMA derivatives on macrophages could be related to molecular weight (Table II), and that for macrophage stimulation is compared in Table I. Among these SMA derivatives, SMABSA in the tested derivatives exhibited the greatest

stimulatory effect on macrophages, although its lytic effect was lower than that of butyl-SMA. Therefore, it was suggested that the lytic action of SMA was reduced by conjugation with a high-molecular-weight protein, such as NCS (12,000) and BSA (69,000), although the stimulatory effect on macrophages remained unchanged.

The time-course study of macrophage stimulation of various macromolecules and PMA indicates (Figs. 1A and 2A) that plant lectins, which are known to interact with cell membranes of these cells, and SMA derivatives showed immediate and linear increases, whereas that of PMA had a short lag period. This difference in time course between PMA and the others may indicate a different mechanism. PMA may involve the subsequent amplification mechanism, perhaps phosphatidyl inositol cascade (30, 31), in contrast to the direct effect on cell membrane.

The release of lysozyme observed in the SMA polymers (Fig. 3D) might also be attributed either to cell membrane and/or lysosome damage or to a process of exocytosis which is usually associated with the respiratory burst (32). Perhaps the binding of SMA through its hydrophobic alkyl side chain to the hydrophobic portions of plasma membrane may lead to the stimulation of macrophages.

Similar to the effects of SMA derivatives on macrophages as described above, a number of surface-active agents, such as deoxycholate (25), digitonin (25), and saponin (33), were also known to exhibit the same properties with respect to the oxidative metabolism of leukocytes.

Our DPH experiments presented here (Fig. 4) showed that SMA derivatives also induced a rapid change in plasma membrane fluidity of macrophages, which paralleled the stimulation of the respiratory burst as seen previously (34, 35). It appears that the two processes are somehow linked and that they take place in similar concentration ranges and have lipophilicity and molecular weight of SMA derivatives (compare Fig. 4 with Figs. 1B and 3A). Thus, it seems reasonable to assume that SMA derivatives may perturb the membrane through its surfactant activity, which would disorganize the structural integrity of the plasma membrane, resulting in activation of NADPH oxidase.

Several polyanions such as pyran (maleic anhydride divinyl ether copolymer), polynucleotides [poly(I) · poly(C)], and dextran sulfate have been shown to be potent immunomodulators and exhibit host-mediated antitumor activity and interferon-inducing capability (36–39). We have evaluated pyran derivatives for the macrophage activation and found no significant effect in our system regardless of whether its carboxyl group is free or esterified (40).

Therefore, the antitumor activities of polyanions reported previously may be explained by the induction of interferon *in vivo,* and subsequently mediate a cascade system of self-defense mechanisms. In this respect smancs and SMABSA may have modes of antitumor activity different from pyran and other polyanions which do not activate macrophage *in vitro.*

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