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Effects of Squalene on the Immune Responses in Mice(II): Cellular and Non-specific Immune Response and Antitumor Activity of Squalene

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Abstract □ Effects of squalene on cellular and non-specific immune responses and antitumor activity in mice were investigated. Cellular and non-specific immunological assay parameters adopted in the present study were delayed-type hypersensitivity reaction and rosette forming cells (RFC) for cellular immunity, activities of natural killer (NK) cells and phagocyte for non-specific immunity. Squalene resulted in marked increases of cellular and non-specific immune functions and enhancement of host resistance to tumor challenge in dose-dependent manner.

Keywords □ Squalene, delayed-type hypersensitivity reaction, rosette forming cell, natural killer cell activity, phagocytic activity, host resistance to tumor challenge.

Considering that a large amount of squalene has been widely used as a health food, it is important to know as much as possible about effects of squalene on the immune system.

Recent studies have reported that squalene may be useful in the treatment of gastric ulcer, rheumatism, hypertension and diseases of bacterial origin[1,2]. Among them effort has been focused on the antitumor activities[3,4] and the immuno-stimulating action of squalene in recent years.

On the antitumor and immuno-stimulating effects of squalene, Ikekawa *et al.*[5] have described that squalene had no cytotoxic activity *in vivo* and *in vitro*, but showed rather high inhibitory effect against tumor growth of sarcoma 180 in mice. Yamawaki *et al.*[6] have found that squalene and squalane have antitumor activities of cell-wall skeleton of *Nocardia rubra* (N-CWS) in both mice and guinea pig and also are useful as the vehicle oils for the prescription adjuvant. Hirai *et al.*[7] indicated that squalene alone had no antitumor effect, but suggested that N-CWS had more potent antitumor effect in the presence of squalene than in its absence. The findings that squalene itself produced an immuno-stimulating action suggest that squalene does not enhance the antitumor action of N-CWS, but is additive to antitumor activity of N-CWS[8]. Kohashi *et al.*[9] have also confirmed that the squalene-type adjuvant was effective as a vehicle oil for the induction of adjuvant polyarthritis and delayed-type hypersensitivity to purified protein derivative (PPD) of tuberculin in rats.

In the previous study, we investigated effects of squalene on humoral immune reponses in mice[10]. However, effects of squalene on cellular immune responses have not been studied in detail. Therefore, the present study was undertaken to investigate effects of squalene on cellular immune responses and antitumor activity.

EXPERIMENTAL METHODS

Experimental animals

Male ICR mice, 5 to 6 weeks of age, weighing 17-21 grams, were used. The experimental animals were housed individually in each cage and acclimatized for at least 7 days prior to use. Animals were maintained as described in the previous report[10].

Materials and treatment

Squalene was purchased from Sigma Chemical Co. (U.S.A) and dissolved in olive oil (Yakuri Pure

Chemicals Co., Japan). Squalene solution (25, 50 and 100 mg/kg, respectively) were orally administered to ICR mice through a zonde once a day for 28 consecutive days.

Non-treated control group and cyclophosphamide-treated control group orally received olive oil (10 ml/kg) alone.

Cyclophosphamide (Sigma Chemical Co., U.S.A.) was dissolved in sterile saline immediately prior to use. ICR mice were treated with a single dose of cyclophosphamide (5 mg/kg *i.p.*) on the second day prior to secondary immunization.

Antigen preparation

Sheep red blood cells (SRBC) collected from a single female sheep were kept at 4℃ in sterile Alsever's solution (pH 6.1).

Methods of antigen preparation were the same as described in the previous report[10].

Immunization

All experimental mice were immunized by intravenous (*i.v.*) injection of 0.1 ml of SRBC suspension (1×10^8 cells/ml) on the fifth day before the last squalene treatment as described by Reed *et al.*[11] and Lake *et al.*[12]. A secondary immunization was performed as described in the previous report[10].

Preparation and inactivation of serum

The blood sample from each mouse was obtained from the carotid artery.

Preparation and inactivation of serum were the same as described in the previous report[10].

Preparation of spleen cells

ICR mice were killed by cervical dislocation and their spleens were removed aseptically. Cell suspensions from the spleens were prepared in complete medium (RPMI-1640 medium supplemented with 100 unit penicillin/ml, 100 μg streptomycin, and 2 mM L-glutamine) by the modified method of Mishell *et al.*[13]. Preparation of spleen cells was the same as described in the previous report[10]. Cell viability was determined by trypan blue exclusion test. Cell viability always exceeded 95% as determined by counting in hemacytometer chambers.

Assay of delayed-type hypersensitivity reactions

Four days after the primary sensitization, footpad reactions were elicited with a challenge of 10^8 SRBC in 0.05 ml into the left hind footpad (LHFP).

Delayed-type hypersensitivity (DTH) reactions were evaluated at 24 hours after challenge by measuring the increase in thickness with a dial gauge caliper as described by Titus *et al.*[14] and Henningsen *et al.*[15].

The percentage increase was calculated as $(T_{24}-T_0)\times100$ per T_0, where T_0 is the thickness of LHFP just before challenge and T_{24} are the thickness of LHPF at 24 hours after challenge.

Assay of resette formation

Rosette formation was functionally determined by the modified method of Gravey *et al.*[16] and Elliott *et al.*[17]. Briefly, spleen cells were suspended in cold-HBSS to the desired concentration (2×10^7 cells/ml). An aliquot of 0.25 ml of spleen cell suspension (2×10^7 cells/ml) was mixed with 0.25 ml of SRBC suspension (2×10^8 cells/ml). The mixed cell suspension was centrifuged at 200×G for 12 minutes. The supernatant was removed and the top layer of the pellet was held at 4℃ for 2 hours, and resuspended by shaking. One drop of resuspended cell suspension was mounted on the a hemacytometer chamber and then covered by a cover glass. One hundred lymphocytes were counted under a phase contrast microscope (Bauch and Lomb, Balphen Microscope) and all lymphocytes binding more than three SRBC were regarded as a rosette formation.

Percent rosette formation was obtained from the equation.

$$\%\text{Rosette} = \frac{\text{Number of rosette forming cells}}{\text{Total cells counted}\times\%\text{ viability}}\times100$$

Preparation of effector cells

Ten mice from every experimental group were sacrificed, and 10 spleens were pooled in each petridish containing 20 ml cold-HBSS. After washout twice with HBSS, spleen cell suspensions were prepared in cold-HBSS by gentle teasing of the organ with forceps, and passing it through nylon mesh to remove major tissue aggregates. After allowing the tissue debris to sediment for 5 minutes on an ice-bath, the cell suspension in HBSS was layered on Ficoll-Hypaque solution (specific gravity 1.078) and centrifuged at 400×G for 30 minutes at 18-20℃.

The mononuclear cell band was harvested, and washed 3 times with HBSS.

All cells were resuspended in complete medium to the desired concentration (2×10^7 cells/m*l*). Complete medium is RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories Co., Grand Island, N.Y.), 100 unit penicillin/m*l*, 100 μg streptomycin/m*l*, and 2 mM glutamine.

Preparation of target cells

YAC-1 cell line, a cell line of Moloney virus induced lymphoma of A/Sn origin, was used as target cells.

This cell line was obtained from the Research Institute of Energy and maintained in complete medium. The target cells were labeled by incubating 2×10^5 cells in 1 m*l* medium with 100 μ Ci of $Na_2{}^{51}CrO_4$ (specific activity 283.58 m*l* Ci/mg, 1 m Ci/m*l*; New England Nuclear) for 1 hr at 37℃ in CO_2 incubator (Queue). The labeled cells were washed three times with HBSS supplemented with 10% fetal bovine serum (10% FBS-HBSS), and adjusted to the desired concentration (2×10^5 cells/m*l*).

Assay of NK cell activity

NK cell activity was measured by using 18 hours ^{51}Cr release assay[18].

Desired concentrations of effector cells were mixed with labeled target cells in 1 m*l* per 96-well flat bottom tissue culture plates (Costar, Cambridge, MA) in triplicate, and incubated for 5 hours at 37℃ in 5% CO_2 in air.

Most experiments were performed with effector to target (E : T) ratios of 100 : 1 and 50 : 1. The plates were centrifuged for 10 minutes 500×G at 4℃, and 100 μ*l* of the supernatants were harvested from each well and were counted in an automatic gamma counter (Beckman, U.S.A.).

The percentage of released isotope was calculated by the following formula:

$$\text{Specific } {}^{51}\text{Cr release}(\%) = \frac{\text{c.p.m. Experimental} - \text{c.p.m. SR}}{\text{c.p.m. MR} - \text{c.p.m. SR}} \times 100$$

where spontaneous release (SR) was defined as the counts per minute (c.p.m.) released from targets incubated with the medium alone, and maximum release (MR) was determined as c.p.m. in the supernatants after lysis of target with 1% Triton x−100. Throughout the experiments, MR was higher than 95% of total isotope uptake, and SR was less than 10%.

Assay of phagocyte activity

Phagocyte activity is based on the work of Biozzi *et al.*[19].

In brief, phagocyte activity was determined on the second day after the last squalene treatment. For the preparation of suspension of carbon, rotring ink was diluted 1/6 with 1% gelatin and kept in a stoppered tube at 37℃ during the experiment. Injection was executed *via* the lateral tail vein by using a 1 m*l* syringe with 26 gauze needle at the dose of 0.01 m*l* of colloidal carbon solution per gram of mouse. In order to dilute the vein, xylene was used. At the interval of 10 min, 20 min and 30 min, 20 μ*l* of blood sample was obtained from the retro-orbital venous plexus. The collected blood samples were expelled into each vial containing 2 m*l* of 0.1% sodium carbonate, and the contents were well mixed for the lysis of erythrocytes. The absorbance of the colloidal carbon contained in blood was measured directly with UV spectrophotometer (Varian, Cary 219) at 600 nm using water as blank. Ten times of density readings were converted to logarithmic scale and plotted against time. The slope of the line is called phagocytic coefficient K. The mice were killed and the weights of spleen and liver were measured.

Corrected phagocytic index which is a measure of phagocytic activity per unit weight of tissue.

$$\text{Corrected phagocytic index} = \frac{\text{WB}}{\text{WS}+\text{WL}} \times \sqrt[3]{\text{K}}$$

*WB, WS and WL are weights of body, spleen, and liver, respectively.

Survival test

Survival test was carried out by the same method as reported previously[5].

The cells of sarcoma 180 were intraperitoneally (*i.p.*) transplanted in ICR ice weekly. The sarcoma 180 cells were obtained aseptically from the ascite of the transplanted mice. Ascites fluid was washed several times with physiological saline. ICR mice

of group 5, 6, 7 and 8 were bearing subcutaneously (*s.c.*) tumor by transplanting 1×10^6 cells of sarcoma 180 on day −21. ICR mice of the squalene-treated groups were orally administered each samples daily for consecutive 18 days starting on day −19 and ending on day −2. Non-treated control and tumor bearing-treated control groups received olive oil (10 ml/kg) alone orally. All of ICR mice were *i.p.* challenged with 1×10^4 cells of sarcoma 180 on day 0, and the survival days of each mouse were estimated.

***Inhibition rate against sarcoma 180 cells*[20]**

All of ICR mice were *s.c.* transplanted with 1×10^6 cells of sarcoma 180 in a volume of 0.1 ml at 24 hours before the start of squalene treatment. Four groups of 10 male mice were divided, one group as control and the others were treated groups. The former was administered olive oil alone orally and the latter was administered each samples orally once daily for 26 consecutive days. Twenty eight days after tumor implantation, the ICR mice were sacrificed and tumors were resected and inhibition rates were calculated from their weights.

The tumor inhibition rate was calculated by the following formula:

$$\text{Relative change}(\%)=\frac{TW}{CW}\times100$$

where CW is a mean value of the tumor weights of the control group and TW is those of the squalene-treated groups.

Statistical analysis

All experimental data were expressed as the mean ± standard error (S.E.).

All data were examined for their statistical significances of differences with Student's t-test[21,22].

RESULTS

Effects of squalene on delayed-type hypersensitivity reaction

Table I shows the effects of squalene on delayed-type hypersensitivity (DTH) reaction in ICR mice. DTH reactions of the squalene (25, 50 and 100 mg/kg)-treated groups were 122.97± 18.73% ($p<0.001$), 122.74± 28.43% ($p<0.001$) and 125.31± 20.51% ($p<0.001$), respectively. The results show significant increase as compared with the corresponding non-treated control group (100%).

Table I. Effects of squalene on delayed-type hypersensitivity (DTH) reaction in ICR mice

Group(mg/kg)	FPSI	Relative change(%)
NC	8.97± 1.25	100
SQ 25	11.03± 1.68***	1.22.97± 18.73***
SQ 50	11.01± 2.55***	122.74± 28.43***
SQ 100	11.24± 1.84***	125.31± 20.51***
SQ 25+CY 5	8.43± 1.08°°°	124.34± 15.93°°°
SQ 50+CY 5	8.41± 1.71°°°	124.04± 10.47°°°
SQ 100+CY 5	8.50± 1.75°°°	125.37± 25.81°°°
CY 5	6.78± 1.16	100

Squalene (SQ; 25, 50 and 100 mg/kg) was orally administered to ICR mice once a day for 28 consecutive days. ICR mice were treated with a single dose of cyclophosphamide (CY; 5 mg/kg *i.p.*) on the second day prior to secondary immunization.

Each value is the mean± S.E. of the results obtained from 10 mice.

Shaded asterisks denote the significance of the difference between the non-treated control (NC) group and the squalene-treated groups; ***, $p<0.001$.

Open asterisks denote the significance of the difference between the squalene plus cyclophosphamide-treated groups and the cyclophosphamide-treated control group; °°°, $p<0.001$.

DTH reactions of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 124.34± 15.93% ($p<0.001$), 124.04± 10.47% ($p<0.001$) and 125.37± 25.81% ($p<0.001$), respectively. The results show significant increase as compared with the cyclophosphamide (5 mg/kg)-treated control group (100%).

These results indicate that the effects of cyclophosphamide on the DTH were significantly prevented by the squalene treatment.

Effects of squalene on rosette forming cells

Table II shows the effects of squalene on rosette forming cells (RFC) in ICR mice. RFC were 116.27 ± 6.78% ($p<0.001$) in the squalene (25 mg/kg)-treated group, 188.14± 13.90% ($p<0.001$) in the squalene (50 mg/kg)-treated group, 234.58± 10.51% ($p<0.001$) in the squalene (100 mg/kg)-treated group. The results show significant enhancement along with the increase of squalene doses as compared with the corresponding non-treated control group (100%).

Table II. Effects of squalene on rosette forming cells (RFC) in ICR mice

Group(mg/kg)	RFC(%)#	Relative change(%)
NC	2.95± 0.52	100
SQ 25	3.43± 0.20***	116.27± 6.78***
SQ 50	5.55± 0.41***	188.14± 13.90***
SQ 100	6.92± 0.31***	234.58± 10.51***
SQ 25+CY 5	3.34± 0.27°°°	153.92± 12.44°°°
SQ 50+CY 5	4.39± 0.33°°°	202.30± 15.21°°°
SQ 100+CY 5	4.01± 0.48°°°	184.79± 22.12°°°
CY 5	2.17± 0.31	100

Mice were challenged with 10^8 SRBC on left hind footpad 4 days after sensitization. On the 5th day, RFC assay was performed.

$$\# \text{ RFC}(\%) = \frac{\text{No. of rosette forming cell}}{\text{Total cell counted} \times \%\ \text{viability}} \times 100$$

Other legends and methods are the same as described in Table I.

Each value is the mean± S.E. of the results obtained from 10 mice.

(***, $p<0.001$ and °°°, $p<0.001$).

RFC of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 153.92± 12.44% ($p<0.001$), 202.30± 15.21% ($p<0.001$) and 184.79± 22.12% ($p<0.001$), respectively. The results show significant increase as compared with the cyclophisphamide (5 mg/kg)-treated control group (100 %).

These results indicate that the effects of cyclophosphamide on the RFC were significantly prevented by the squalene treatment.

Effects of squalene on natural killer cell activity

Table III shows the effects of squalene on natural killer (NK) cell activity in mice.

In the case of the effector to target cells ratio was 100 : 1, NK cell activity was 110.10± 13.95% ($p<0.001$) in the squalene (25 mg/kg)-treated group, 119.05± 19.95% ($p<0.001$) in the squalene (50 mg/kg)-treated group, 130.14± 14.62% ($p<0.001$) in the squalene (100 mg/kg)-treated group.

The results show significant enhancement along with the increase of squalene doses as compared with the corresponding non-treated control group (100%).

NK cell activities of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 122.55± 18.25% ($p<0.001$), 114.21± 13.71% ($p<0.001$) and 118.38± 10.72% ($p<0.001$), respectively. The results show significant increase as compared with the cyclophosphamide (5 mg/kg)-treated control group (100%).

In the case of the effector to target cell ratio was 50 : 1, NK cell activity was 112.36± 12.92% ($p<0.001$) in the squalene (25 mg/kg)-treated group, 115.78 ± 16.14% ($p<0.001$) in the squalene (50 mg/kg)-treated group, 118.08± 13.13% ($p<0.001$) in the squalene (100 mg/kg)-treated group.

The results show significant enhancement along with the increase of squalene doses as compared with the corresponding non-treated control group (100%).

NK cell activities of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 121.61± 21.54% ($p<0.001$), 114.60± 19.30% ($p<0.001$) and 102.88± 9.08% ($p<0.001$), respectively. The results show significant enhancement as compared with the cyclophosphamide (5 mg/kg)-treated control group (100%).

Effects of squalene of phagocyte activity

Table IV shows the effects of squalene on corrected phagocytic index in ICR mice.

Corrected phagocytic index was 105.24± 9.58% ($p<0.001$) in the squalene (25 mg/kg)-treated group, 112.12± 6.33% ($p<0.001$) in the squalene (50 mg/kg)-treated group, 108.32± 3.07% ($p<0.001$) in the squalene (100 mg/kg)-treated group. The results show significant enhancement as compared with the corresponding non-treated control group (100%). Corrected phagocytic indexes of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 100.99± 8.12%, 107.33± 12.08% ($p<0.001$) and 113.27± 10.49% ($p<0.001$), respectively. Especially, corrected phagocytic indexes of the squalene at 50 and 100 mg/kg plus cyclophosphamide-treated groups were significantly enhanced along with the increase of squalene doses ad compared with the cyclophosphamide (5 mg/kg)-treated control group (100%).

These results indicate that the effects of cyclophosphamide on the reticuloendothelial system (phagocytic function) were significantly suppressed by the high dose of squalene treatment.

Table III. Effects of squalene on natural killer cell activity in ICR mice

Group (mg/kg)	% Specific lysis of ^{51}Cr-labelled target cell(*YAC*-1) #			
	E : T//100 : 1	Relative change(%)	E : T//50 : 1	Relative change(%)
NC	21.00± 2.04	100	19.58± 2.77	100
SQ 25	23.33± 2.93***	111.10± 13.95***	22.00± 2.53***	112.36± 12.92***
SQ 50	25.00± 4.19***	119.05± 19.95***	22.67± 3.16***	115.78± 16.14***
SQ 100	27.33± 3.07***	130.14± 14.62***	23.12± 2.57***	118.08± 13.13***
SQ 25+CY 5	19.67± 2.93°°°	122.55± 18.25°°°	17.33± 3.07°°°	121.61± 21.54°°°
SQ 50+CY 5	18.33± 2.20°°°	114.21± 13.71°°°	16.33± 2.75°°°	114.60± 19.30°°°
SQ 100+CY 5	19.00± 1.72°°°	118.38± 10.72°°°	16.66± 1.47°°°	102.88± 9.08°°°
CY 5	16.05± 2.63	100	14.25± 2.79	100

Effector : Target cell(E : T)

The % lysis was determined by a standard 4 hours ^{51}Cr release assay and effector to target ratios were 100 : 1 and 50 : 5.

Other legends and methods are the same as described in Table I.

Each value is the mean± S.E. of the results obtained from 10 mice.

(***, $p<0.001$ and °°°, $p<0.001$)

Table IV. Effects of squalene on phagocyte activity in ICR mice

Group (mg/kg)	Corrected phagocytic index	Relative change(%)
NC	5.53± 0.40	100
SQ 25	5.82± 0.53***	105.24± 9.58***
SQ 50	6.20± 0.35***	112.12± 6.33***
SQ 100	5.99± 0.17***	108.32± 3.07***
SQ 25+CY 5	5.10± 0.41	100.99± 8.12
SQ 50+CY 5	5.42± 0.61°°°	107.33± 12.08°°°
SQ 100+CY 5	5.72± 0.53°°°	113.27± 10.49°°°
CY 5	5.05± 0.50	100

Corrected phagocytic index is a constant obtained from a formula relating the cube root K to the ratio of body weight to the weight of the liver and spleen.

Other legends and methods are the same as described in Table I.

Each value is the mean± S.E. of the results obtained from 10 mice.

(***, $p<0.001$ and °°°, $p<0.001$)

Effects of squalene on survival rate of ICR mice bearing sarcoma 180 cells

Table V shows the effects of squalene on survival rate of ICR mice bearing sarcoma 180 cells.

Survival rates of the squalene (25, 50 and 100 mg/kg)-treated groups were significantly enhanced by 106.7± 6.72% ($p<0.001$), 117.2± 13.44% ($p<0.001$) and 129.8± 12.11% ($p<0.001$) of each non-treated control group (100%), respectively.

On the other hand, survival rates of the squalene at 25, 50 and 100 mg/kg plus tumor bearing-treated groups were 129.9± 23.89% ($p<0.001$), 164.2± 25.38% ($p<0.001$) and 222.4± 13.43% ($p<0.001$), respectively. The results show significant enhancement as compared with the tumor bearing-treated control group (100%).

It is noteworthy that squalene has a considerable host's resistance against challenged ascites sarcoma 180.

Effects of squalene on the growth of sarcoma 180 ascite tumor

Table VI shows the effects of squalene on growth of sarcoma 180 ascite tumor in ICR mice.

Growths of sarcoma 180 ascite tumor of the squalene (25, 50 and 100 mg/kg)-treated groups were significantly depressed by 39.12± 7.16% ($p<0.001$), 37.74± 11.85% ($p<0.001$) and 34.44± 6.34% ($p<0.001$) of each non-treated control group (100%), respectively.

These results indicated that the increase of squalene doses showed strong antitumor activity against sarcoma 180 ascite tumor.

DISCUSSION

Delayed type hypersensitivity (DTH) response[23-25] remains the standard means of clinically assessing *in vivo* cell-mediated immune status. In the present study, squalene displayed enhanced DTH reactions that were significantly different from the non-treated

Table V. Effects of squalene on servival of ICR mice transplanted with sarcoma-180 tumor

Experimental group	Treatment	Mean survival days	T/C(%)	No. of 50 days survivals
				No. in group
1	NC	23.8± 3.2	100	1/10
2	SQ 25 mg/kg	25.4± 1.6***	106.7± 6.72***	0/10
3	SQ 50 mg/kg	27.9± 3.2***	117.2± 13.44***	0/10
4	SQ 100 mg/kg	30.9± 2.9***	129.8± 12.11***	1/10
5	SQ 25 mg/kg+Tumor-bearing	8.7± 1.6°°°	129.9± 23.89°°°	0/10
6	SQ 50 mg/kg+Tumor-bearing	11.0± 1.7°°°	164.2± 25.38°°°	0/10
7	SQ 100 mg/kg+Tumor-bearing	14.9± 0.9°°°	222.4± 13.43°°°	0/10
8	Tumor-bearing	6.7± 1.6	100	0/10

ICR mice of group 5, 6, 7 and 8 were bearing *s.c.* tumior by tumor by transplanting 1×10^6 cells of sarcoma 180 on day −21. ICR mice of the squalene-treated groups were administered each samples orally 18 times on day −19···−2. Non-treated control and tumor bearing-treated control groups received olive oil (10 ml/kg) alone orally.

All of the ICR mice were *i.p.* challenged with 1×10^4 cells of sarcoma 180 on day 0, and the survival days of each mouse were estimated.

Each value is the mean± S.E. of the results obtained from 10 mice.

Shaded asteriks dedote the significance of the difference between the non-treated control group and the squalene-treated groups; ***, $p<0.001$. Open asterisks denote the significance of the difference between the squalene plus tumor bearing-treated groups and the tumor-bearing-treated control group; °°°, $p<0.001$.

control group as shown in Table I. It was shown that squalene treatment significantly enhanced DTH reaction.

Rosette technique had been widely used for the detection of antibody formation *in vitro* by isolated spleen cells. Both T and B cells have been shown to be rosette forming cells. In addition, macrophages can form rosettes with SRBC possibly *via* cytophilic anti-erythrocyte antibody[26]. However, it is known that rosette forming cells (RFC) are associated with T cells mainly. In the present study, it was showed that RFC were significantly enhanced by the squalene treatment (Table II). And these results are similar to those of the previous studies. For example, Offner *et al.*[27] reported that unsaturated fatty acids enhanced E rosette formation by T cells. The finding that highly purified squalene (HP-SQ) enhanced DTH in the healthy mice suggest that HP-SQ intensify DTH inducing cells activity, *i.e.* T-lymphocytes function, of the healthy and the tumor-bearing hosts [8]. From the above results of DTH and RFC, these data suggest that squalene may enhance the cellular immune responses not by depression of antibody formation, but by enhancement of T-lymphocyte activity, c-GMP activity and helper T cells induction.

In the present study, NK cell activity was significantly enhanced along with the increase of squalene doses (Table III). These results seemed to suggest that the enhancing effect of squalene on the NK cell activity may be due to the production of lymphokine by activated lymphocytes.

The corrected phagocytic index was significantly enhanced by the squalene treatment (Table IV). And these results are in accordance with the host's immunologic observation reported by Ohkuma *et al.*[8] In view of their report, it is thought that squalene may stimulate the fixed phagocytic cells of the reticuloendothelial system (RES).

While cyclophosphamide, a compound yielding a number of alkylating metabolites after activation by a microsomal enzyme system in the liver[28,29], has considerable anti-neoplastic activity against a variety of experimental tumors and human cancers[30,31], and is one of the most potent immuno-suppressive agents[32]. In the present study, it was shown that suppressive effects of cyclophosphamide on the cell-mediated immune responses were in accordance with the immunologic observation reported by Ahn *et al.*[33] and Oh[34]. The squalene plus cyclophosphamide-treated groups decreased the cellular immune responses in comparison with those of non-treated control group, but significantly enhanced the cellu-

Table VI. Effects of squalene on growth of sarcoma 180 in ICR mice

Group (mg/kg)	Tumor weight (g/mouse)	Relative change # (%)
NC	3.63± 0.67	100
SQ 25	1.42± 0.26***	39.12± 7.16***
SQ 50	1.37± 0.43***	37.74± 11.85***
SQ 100	1.25± 0.23***	34.44± 6.34***

ICR mice of the squalene-treated groups were administered each samples orally and non-treated control group received olive oil (10 ml/kg) alone orally once a day for 26 consecutive days after tumor inoculation. All of ICR mice were *s.c.* transplanted with 1×10^6 cells of sarcoma 180 and tumors were resected and weighed on 28th day after tumor inoculation.

Each value is the mean± S.E. of the results obtained from 10 mice.

Asterisks denote a significant reduction from the corresponding non-treated control (NC) group; ***, $p<0.001$

lar immune responses in comparison with those of the cyclophosphamide (5 mg/kg) treated-control group. These results seemed to suggest that the suppressive effects of cyclophosphamide on the cellular immune responses were significantly prevented by the squalene treatment.

The survival rates of the squalene treatment was extended more 1.5 to 2 time after *i.p.* challenging of sarcoma 180 cells than the non-squalene treatment (Table V). Moreover, Ikekawa *et al.*[5] reported that oral administration of highly purified squalene exerted antitumor activity. From the above report, these results mean that squalene is expected to prevent the death caused by sarcoma 180 cells in ICR mice.

Table VI shows that the squalene treatment significantly inhibited the growth of sarcoma 180 cells *in vivo* as compared with that of the non-treated control group. These results are similar to those of the previous studies[35,36]. From the above reports, these results suggest that antitumor activities of squalene in ICR mice may be related to the augmentation of NK cell activity.

In conclusion, the squalene treatment resulted in enhancement of immune functions such as cellular immunity, NK cell activity, and macrophage function and enhancement of antitumor activities. To elucidate the mechanism of action of these squalene, further studies are in progress.

LITERATURE CITED

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