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Effects of Squalene on the Immune Responses in Mice(I): Humoral Immune Responses of Squalene

Young Keun Ahn and Joung Hoon Kim
College of Pharmacy, Won Kwang University, Iri, 570-749, Korea
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Abstract □ Effects of squalene on humoral immune system in mice were investigated. Squalene exhibited significant increases in the circulating leukocyte counts and relative spleen and thymus weights of the mice. However, the relative liver weight was slightly decreased. Hemagglutination titers (HA) were signficantly enhanced by squalene while Arthus reaction was not affected. Splenic plaque forming cells (PFC) were also greatly increased by squalene, especially at doses of 50 and 100 mg/kg of it.

Keywords □ Squalene, circulating leukocyte count, hemagglutination titer, Arthus reaction, splenic plaque forming cell.

It is well known that squalene, an acyclic triterpene, is a crucial precursor in the biosynthesis of sterols[1,2] which appear to be important components of animal and plant membrances, and also a widely distributed lipid in common western foods, especially in fish and plant oils[3-9].

Recent studies have reported that squalene may be useful in the treatment of gastric ulcer, rheumatism, hypertension and diseases of bacterial origin[10,11].

With regard to the antimicrobial effects of squalene, Masuda *et al.*[12] reported that antifungal activity of amphotericin B on *Saccharomyces cervisiae* was synergistically enhanced when combined with squalene. Heller *et al.*[13] reported that partally purified lipid extracted from a shark liver stimulated host resistance against bacterial infection.

In squalene-related studies on the serum lipids, it is known that dietary squalene can affect the serum level of squalene in man[14,15] and cholesterol concentration of the rat liver[16]. However, the metabolic fate of dietary squalene has not been investigated in detail. Tilvis *et al.*[17] have shown that absorbed dietary squalene contributes to some extent to the squalene content of adipose tissue, effectively increases the overall cholesterol synthesis and also enhances cholesterol elimination preferentially as fecal bile acids in the rat. However, Ichikawa *et al.*[18] reported that oral administration of squalene decreased HDL-cholesterol and increased the thiobarbiturate reactive substances value (TBA value) and phospholipid in serum and liver of rats.

As aforementioned, although numerous studies of squalene on the function of living organisms have been reported, effect of squalene on humoral immune responses have not been studied in detail. Therefore, the present study was undertaken to investigate the humoral immune effects of squalene in mice.

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. The cage were maintained at 23± 2℃ and 50-60% relative humidity throughout the whole experimental period. All experimental mice were fed with animal chows (Jeil Ind. Ltd) and tap water *ad libitum* but deprived of the animal chows for 16 hours prior to sacrifice.

Materials and treatment

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

Chemicals Co., Japan). Squalene solutions (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 received olive oil (10 ml/kg) alone orally.

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.

Lymphoid organ and body weights

The body weights of mice in each group were measured every week throughout the whole experimental period and sacrificed by cervical dislocation on the second day after the last squalene treatment. Liver, spleen and thymus were removed and weighed. Lymphoid organ weight ratio to body weight is calculated for each of mice.

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). SRBC were washed three times with phospnate buffered saline (PBS, Gibco Laboratories Co., Grand Island, N.Y. pH 7.4) after centrifugation 400×G for 10 minutes and adjusted to provide a desired concentration by hemacytometer count (Bright-Line, ® U.S.A.).

Immunization

All experimental mice were immunized by intravenous (*i.v.*) injection of 0.1 m*l* of SRBC suspension (1×10^8 cells/m*l*) on the fifth day before the last squalene treatment as described by Reed *et al.*[19] and Lake *et al.*[20]. A secondary immunization was performed by subcutaneous (*s.c.*) injection of 0.05 m*l* of SRBC suspension (2×10^9 cells/m*l*) into the left hind footpad of mice on the fourth day after the primary sensitization.

Preparation and inactivation of serum

The blood sample from each mouse was obtained from the carotid artery. The blood was allowed to clot in polyethylene tubes at 4℃ for additional 30-60 minutes, and then centrifuged at 700×G for 20 minutes. The serum was withdrawn and heat-inactivated in polyethylene tubes at 56℃ for additional 30 minutes.

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/m*l*, 100 μg streptomycin, and 2 mM L-glutamine) by the modified method of Mishell *et al.*[21]. In brief, the spleens were then minced, and gently squeezed into fragements between the frosted ends of two sterile microscope slides in cold complete medium. The cell suspension was passed through nylon mesh to remove major tissue aggregates. The erythrocytes were lysed with 0.83% ammonium chloride solution. The cell suspension was washed three times by centrifugation and finally suspended in cold complete medium. Cell viability was determined by trypan blue exclusion test. Cell viability always exceeded 95% as determined by counting in hemacytometer chambers.

Hemagglutination (HA) and 2-mercaptoethanol (2-ME) resistant HA titers[22-24]

HA titer was determined in microtitration trays (Limbro Chemical Co., Inc. New Haven, Connecticut) using 0.025 m*l* volume of diluent by serial dilution of inactivated pooled sera in Hank's balanced salt solution (HBSS; Gibco Laboratories Co., Grand Island, N.Y.), which was added on to 0.05 m*l* volume of 0.5% peaked SRBC. For HA titer assay, the plate (Flow Lab., U.S.A.) of serum and SRBC mixture was incubated for 18 hours at 37℃.

HA titer assay in the serum treated with 2-ME was the same method as described previously except that the serum is diluted with HBSS containing 0.15 N 2-ME instead of HBSS alone. Each titration was performed in duplicate and the mean titer was expressed as $\log_2$.

Assay of plaque forming cells (PFC)

In order to examine whether this squalene accelerates the antibody production to heterologous antigen or not, the slide technique of Cunningham *et al.*[25] was utilized. Two days after the last squalene treatment, the spleens were removed from the mice and the number of plaques formed by the antibody forming cell from spleen cells was counted by the

conventional method. In brief, the liqud layer containing the cell suspension is mixed in plastic microtiter plates as follows: a) 650 μl of spleen cell suspension, b) 50 μl of 20% SRBC, and c) 100 μl of complement (Gibco, Laboratories Co., Grand Island, N.Y.) dilution of guinea pig. The above cell suspension is mixed and placed into both chambers of a set of with a pasteur pipette. The chambers are sealed by carefully dipping the slide edges into a melted mixture of equal portions of paraffin and wax. The chambers were incubated for 1 hour in a humidified CO_2 incubator (Quene ®) gassed with 5% CO_2 and 95% O_2 at 37℃ before scoring the plaques under a dissecting microscope. Plaques should be counted within 4 hours after development since after a longer time lapse the plaques tend to become unclear due to movement of cells in the liquid layer.

The plaques are expressed either as PFC per 10^6 nucleated cells or as PFC per total spleen cells.

The plaques were calculated by the following formula;

$$\text{PFC}/10^6 \text{ spleen cells} = \frac{N}{C \cdot V_m \cdot a} \times 10^6$$

$$\text{PFC/total spleen cells} = \left(\frac{\text{PFC}}{10^6 \text{ spleen cells}}\right) \cdot C \cdot V_s$$

$$a = \frac{650}{800}$$

: The ratio of spleen cell suspension in cultured mixture.

N : The number of plaque observed in chamber.

C : The count of spleen cells in 1 m*l* of spleen cell suspension.

V_m: Volume of incubation mixture filled into a chamber (m*l*).

V_s : Total volume of spleen cell suspension (m*l*)

Assay of Arthus reaction

Four days after the primary sensitization, footpad reaction was elicited with a challenge of 10^8 SRBC in 0.05 m*l* into the left hind footpad (LHFP). The footpad reaction was evaluated at 3 hours after challenge by measuring the increase in thickness with a dial gauge caliper as described by Titus *et al.*[26] and Henningsen *et al*[27].

The percentage increase was calculated as $(T_3 - T_0) \times 100$ per T_0, where, T_0 is the thickness of LHFP just challenge and T_3 is the thickness of LHPF at 3 hours after challenge.

Assay of circulating leukocyte[28]

Blood samples for measuring leukocyte in ICR male mice were collected from the retro-orbital venous plexus on the second day after the last squalene treatment. Türk's solution was used for staining leukocytes and lysis of unnucleated cells. The number of nucleated cells was counted in hemacytometer chamber under a microscope. Triple counting per sample was carried out and the mean value of the results was calculated. The number was compared with that obtained from control mice.

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[29,30].

RESULTS

Effects of squalene on body and relative organ weights

Table I shows the body weight of mice. The body weights of the squalene (25 and 50 mg/kg)-treated groups showed 86.83± 8.25% ($p<0.001$) and 90.94± 9.34% ($p<0.001$) of each non-treated control group (100%), respectively, while the body weights of the squalene (100 mg/kg)-treated group showed 104.19± 14.86% of the corresponding non-treated control group. The body weights of the squalene at 25, 50 and 100 mg/kg plus cyclophospohamide-treated groups showed 132.70± 16.00% ($p<0.001$), 136.90± 18.69% ($p<0.001$) and 157.48± 16.89% ($p<0.001$) of each cyclophosphamide (5 mg/kg)-treated control group (100%), respectively.

Table II shows the liver weights and the weight ratios of liver to body weight. The squalene (25, 50 and 100 mg/kg)-treated groups showed 99.64± 7.09%, 98.73± 5.09% and 98.18± 6.54% of the corresponding non-treated control group (100%), respectively, but there was non-significant difference by comparing it with the corresponding non-treated control group (100%).

On the other hand, the weight ratios of liver to body weight of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups were significantly decreased to 91.89± 5.72% ($p<0.001$), 80.92± 1.

Table I. Effects of squalene on the body weight of ICR mice

Group (mg/kg)	Increasing rate (%)	Relative change (%)
NC	39.63± 5.17	100
SQ 25	34.41± 3.29***	86.83± 8.25***
SQ 50	36.04± 3.70***	90.94± 9.34***
SQ 100	41.29± 5.89	104.19± 14.86
SQ 25+CY 5	28.12± 3.39○○○	132.70± 16.00○○○
SQ 50+CY 5	29.01± 3.96○○○	136.90± 18.69○○○
SQ 100+CY 5	33.37± 3.58○○○	157.48± 16.89○○○
CY 5	21.19± 2.60	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 30 mince.

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; ○○○, $p<0.001$.

Table II. Effects of squalene on the liver weight of ICR mice

Group (mg/kg)	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$	Relative change (%)
NC	5.50± 0.43	100
SQ 25	5.48± 0.39	99.64± 7.09
SQ 50	5.43± 0.28	98.73± 5.09
SQ 100	5.40± 0.36	98.18± 6.54
SQ 25+CY 5	5.78± 0.36○○○	91.89± 5.72○○○
SQ 50+CY 5	5.09± 0.11○○○	80.92± 1.75○○○
SQ 100+CY 5	5.28± 0.16○○○	83.94± 2.54○○○
CY 5	6.29± 0.53	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.

(○○○, $p<0.001$)

75% ($p<0.001$) and 83.94± 2.54% ($p<0.001$) of each cyclophosphamide (5 mg/kg)-treated control group (100%), respectively.

Table III shows the spleen and thymus weights of mice.

In the spleen weight, the weight ratios of spleen to body weight of the squalene (25, 50 and 100 mg/kg)-treated groups showed 106.45± 9.68% ($p<0.01$), 112.90± 27.95% ($p<0.01$) and 113.98± 4.30% ($p<0.001$) of each non-treated control group (100%), respectively, and there was significant difference by comparing it with the corresponding non-treated control group.

The weight ratios of spleen to body weight of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 139.62± 16.99% ($p<0.001$), 132.08± 11.31% ($p<0.001$) and 133.96± 7.55% ($p<0.001$), respectively. The results show significant increase as compared with those of the cyclophosphamide (5 mg/kg)-treated control group (100%).

In the weight ratios of thymus to body weight, in comparison with the corresponding non-treated control group (100%), squalene (25 mg/kg)-treated group significantly decreased the weight ratio of thymus to body weight (88.24± 5.88%, $p<0.001$), but the weight ratios of thymus to body weight of squalene (50 and 100 mg/kg)-treated groups showed 105.88± 11.76% ($p<0.001$) and 129.41± 17.65% ($p<0.001$), respectively. The results show significance statistical.

The weight ratios of thymus to body weight of squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 140.00± 20.00% ($p<0.001$), 140.00± 20.00% ($p<0.001$) and 170.00± 20.00% ($p<0.001$), respectively, the results of which were significantly increased as compared with those of cyclophosphamide (5 mg/kg)-treated control group (100 %).

Effects of squalene of hemagglutination (HA) titer and 2-ME resistant (2MER) HA titer

Table IV shows the effects of squalene on HA

Table III. Effects of squalene on the spleen and thymus weights on ICR mice

Group(mg/kg)	$\frac{\text{Spleen wt.}}{\text{Body wt.}} \times 100$	Relative change(%)	$\frac{\text{Thymus wt.}}{\text{Body wt.}} \times 100$	Relative change(%)
NC	0.93± 0.16	100	0.17± 0.01	100
SQ 25	0.99± 0.09**	106.45± 9.68**	0.15± 0.01***	88.24± 5.88***
SQ 50	1.05± 0.26**	112.90± 27.95**	0.18± 0.02***	105.88± 11.76***
SQ 100	1.06± 0.04***	113.98± 4.30***	0.22± 0.03***	129.41± 17.65***
SQ 25+CY 5	0.74± 0.09$^{\circ\circ\circ}$	139.62± 16.99$^{\circ\circ\circ}$	0.14± 0.02$^{\circ\circ\circ}$	140.00± 20.00$^{\circ\circ\circ}$
SQ 50+CY 5	0.70± 0.06$^{\circ\circ\circ}$	132.08± 11.31$^{\circ\circ\circ}$	0.14± 0.02$^{\circ\circ\circ}$	140.00± 20.00$^{\circ\circ\circ}$
SQ 100+CY 5	0.71± 0.04$^{\circ\circ\circ}$	133.96± 7.55$^{\circ\circ\circ}$	0.17± 0.02$^{\circ\circ\circ}$	170.00± 20.00$^{\circ\circ\circ}$
CY 5	0.53± 0.09	100	0.10± 0.01	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. (**, $p<0.01$, ***, $p<0.001$ and $^{\circ\circ\circ}$, $p<0.001$).

Table IV. Effects of squalene on antibody production in ICR mice

Group(mg/kg)	HA titer (log_2)#	Relative change(%)	MER-HA titer (log_2)#	Relative change(%)
NC	3.40± 0.17	100	2.40± 0.17	100
SQ 25	3.20± 0.14***	94.12± 4.12***	2.41± 0.28	100.42± 11.67
SQ 50	4.60± 0.17***	135.29± 5.00***	3.40± 0.17***	141.67± 7.08***
SQ 100	4.80± 0.27***	141.18± 7.94***	2.40± 0.17	100.00± 7.08
SQ 25+CY 5	2.60± 0.17$^{\circ\circ\circ}$	92.86± 6.07$^{\circ\circ\circ}$	2.20± 0.14$^{\circ\circ\circ}$	157.14± 18.00$^{\circ\circ\circ}$
SQ 50+CY 5	2.60± 0.17$^{\circ\circ\circ}$	92.86± 6.07$^{\circ\circ\circ}$	2.20± 0.22$^{\circ\circ\circ}$	142.86± 15.72$^{\circ\circ\circ}$
SQ 100+CY 5	2.40± 0.17$^{\circ\circ\circ}$	85.71± 6.07$^{\circ\circ\circ}$	1.60± 0.17$^{\circ\circ\circ}$	114.29± 12.14$^{\circ\circ\circ}$
CY 5	2.80± 0.05	100	1.40± 0.15	100

HA : Hemagglutination MER-HA : 2-Mercaptoethanol resistant HA.
Mice were challenged with 10^8 SRBC 4 days after sensitization.
On the 5th day, HA and MER-HA titers were assayed.
Other legends and methods are the same as described in Table I.
Each value is the mean± S.E. (log_2) of the results obtained from 10 mice.
(***, $p<0.001$ and $^{\circ\circ\circ}$, $p<0.001$).

titer and 2 MER HA titer in ICR mice.

HA titer of the squalene (25 mg/kg)-treated group showed 94.12± 4.12% ($p<0.001$) in comparison with that of the corresponding non-treated control group (100%). HA titers of the squalene (50 and 100 mg /kg)-treated groups showed 135.29± 5.00% ($p<0.001$) and 141.18± 7.94% ($p<0.001$), respectively. The results show significant increase as compared with those of the corresponding non-treated control group.

HA titers of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 92.86 ± 6.07% ($p<0.001$), 92.86± 6.07% ($p<0.001$) and 85.71 ± 6.07% ($p<0.001$), respectively. The results show significant decrease as compared with those of the cyclophosphamide (5 mg/kg)-treated control group (100%).

2-MER HA titers of the squalene (50 mg/kg)-treated group showed 141.67± 7.08% ($p<0.001$) as compared with those of the corresponding non-treated control group (100%). The results show significant increase.

2-MER HA titers of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 157.14± 18.00% ($p<0.001$), 142.86± 15.72% ($p<0.001$) and 114.29± 12.14% ($p<0.001$), 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 the cyclophosphamide on the antibody production were ge-

Table V. Effects of squalene on hemolytic plaque forming cell (PFC) in ICR mice

Group(mg/kg)	PFC/10^6 spleen cells	Relative change(%)	PFC/spleen ($\times 10^2$)	Relative change(%)
NC	64.33± 8.53	100	78.67± 6.64	100
SQ 25	71.12± 7.36***	110.55± 11.44***	80.33± 11.15	102.11± 14.17
SQ 50	70.00± 8.40***	108.81± 13.06***	95.33± 16.77***	121.18± 21.32***
SQ 100	77.67± 7.12***	120.74± 11.07***	261.67± 23.44***	332.62± 29.80***
SQ 25+CY 5	60.33± 7.15ooo	163.05± 19.32ooo	71.33± 8.94ooo	248.80± 31.18ooo
SQ 50+CY 5	42.33± 8.27ooo	114.41± 22.35ooo	46.33± 7.36ooo	161.60± 25.67ooo
SQ 100+CY 5	59.33± 8.93ooo	160.35± 24.14ooo	68.00± 10.81ooo	237.18± 37.70ooo
CY 5	37.00± 6.14	100	28.67± 5.65	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 $^{ooo}p<0.001$).

nerally prevented by squalene treatment.

Effects of squalene on plaque forming cells

Table V shows the effects of squalene on the number of plaque forming cells in ICR mice.

In the number of plaque forming cells (PFC) per 10^6 spleen cells of ICR mice, PFC of the squalene (25, 50 and 100 mg/kg)-treated groups showed 110.55 ± 11.44% ($p<0.001$), 108.81± 13.06% ($p<0.001$) and 120.74± 11.07% ($p<0.001$), respectively. The results show significant increase as compared with the corresponding non-treated control group (100%).

PFC of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 163.05± 19.32% ($p<0.001$), 114.41± 22.35% ($p<0.001$) and 160.35± 24.14% ($p<0.001$), respectively. The results show significant increase as compared with the cyclophosphamide (5 mg/kg)-treated control group (100 %).

In the number of PFC per total spleen cells of ICR mice, the number of PFC was significantly enhanced along with the increase of squalene doses as compared with that of the corresponding non-treated control group (100%). That is, the squalene (25, 50 and 100 mg/kg)-treated groups showed 102.11 ± 14.17%, 121.18± 21.32% ($p<0.001$) and 332.62± 29.80% ($p<0.001$), respectively.

PFC of the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 248.80± 31.18% ($p<0.001$), 161.60± 25.67% ($p<0.001$) and 237.18± 37.70% ($p<0.001$), respectively. The results show significant increase as compared with the cyclophosphamide (5 mg/kg)-treated control group (100 %).

These increases in PFC number of the squalene treatment indicate the possibility that squalene significantly restored the suppressed antibody production in the cyclophosphamide treatment.

Effects of squalene on Arthus reaction

As shown in Tablve VI, Arthus reactions of the squalene (25, 50 and 100 mg/kg)-treated groups showed 104.91± 19.65%, 102.68± 12.83% and 105.30± 23.36%, respectively. The results show slight enhancement as compared with the corresponding non-treated control group (100%).

In comparison with the cyclophosphamide (5 mg/kg)-treated control group (100%), the squalene at 25, 50 and 100 mg/kg plus cyclophosphamide-treated groups showed 103.14± 17.31%, 100.88± 22.38 % and 101.24± 12.64%, respectively, but there was non-significant difference by comparing it with the cyclophosphamide (5 mg/kg)-treated control group.

Effects of squalene on number of circulating leukocytes

Table VII shows the effects of squalene on number of circulating leukocytes in ICR mice.

The number of the circulating leukocytes was 112.62± 21.19% ($p<0.001$) in the squalene (25 mg/kg)-treated group, 117.98± 18.71% ($p<0.001$) in the squalene (50 mg/kg)-treated group, 118.87± 20.70% ($p<0.001$) 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%).

The numbers of circulating leukocytes of the squalene at 25, 50 and 100 mg/kg plus cyclophos-

Table VI. Effects of squalene on Arthus reaction in ICR mice

Group (mg/kg)	FPSI	Relative change (%)
NC	15.67± 1.99	100
SQ 25	16.44± 3.08	104.91± 19.65
SQ 50	16.09± 2.01	102.68± 12.83
SQ 100	16.50± 3.66	105.30± 23.36
SQ 25+CY 5	14.12± 2.37	103.14± 17.31
SQ 50+CY 5	13.81± 1.05	100.88± 22.38
SQ 100+CY 5	13.86± 1.73	101.24± 12.64
CY 5	13.69± 1.37	100

Mice were challenged with 10^8 SRBC on left hind footpad 4 days after sensitization. Footpad thickness was measured immediately before challenge and 3 hr after challenge.

Footpad swelling index(FPSI)= $\frac{T_3-T_0}{T_0}\times 100$

where, T_0 is the left hind footpad thickness immediately before challenge and T_3 is the left hind foodpad thickness 3 hr after challenge.

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.

Table VII. Effects of squalene on number of circulating leukocytes in ICR mice

Group (mg/kg)	Number of circulating leukocytes (/mm^3)	Relative change(%)
NC	6.720± 1.127	100
SQ 25	7.568± 1.424***	112.62± 21.19***
SQ 50	7.928± 1.257***	117.98± 18.71***
SQ 100	7.988± 1.391***	118.87± 20.70***
SQ 25+CY 5	4.992± 691$^{○○}$	109.47± 15.15$^{○○}$
SQ 50+CY 5	5.520± 648$^{○○○}$	121.05± 14.21$^{○○○}$
SQ 100+CY 5	6.040± 868$^{○○○}$	132.46± 19.04$^{○○○}$
CY 5	4.560± 834	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.

(***, $p<0.001$, $^{○○}$, $p<0.01$ and $^{○○○}p<0.001$).

phamide-treated groups showed 109.47± 15.15% ($p<0.01$), 121.05± 14.21% ($p<0.001$) and 132.46± 19.04% ($p<0.001$), respectively. The results show significant enhancement along with the increase of squalene doses as compared with the cyclophosphamide (5 mg/kg)-treated control group (100%).

These results indicate that the effects of cyclophosphamide on the leukopenia were significantly suppressed by the squalene treatment.

DISCUSSION

The body weight and the ratio of liver to body weight were generally decreased by the squalene treatment compared with the non-treated control group (Table I and II). The present experimental data were similar to those of the previous reports[17, 18]. In view of these reports, it is believed that squalene may reduce the body weight by the enhancement of lipids metabolism.

The ratios of spleen and thymus to body weight were significantly increased along with the increase of squalene doses (Table III). In view of Wachsmuch's report[31], these increases in the relative immuno-organ weight suggested that immune function might be enhanced dose-dependently.

The hemagglutination test detects antibodies to antigens as an antigen-antibody reaction to SRBC. By performing these reactions *in vitro* it may be exploited in order to determine the amount of antibody present in serum. In the present study, hemagglutanation (HA) titer and 2 MER-HA titer were significantly enhanced along with the increase of squalene doses (Table IV). These results indicate that the humoral immune responses might be enhanced by the increase of squalane doses.

Splenic plaque forming cell assays are used to detect antibody forming cells. In the present study, splenic plaque forming cells were significantly enhanced along with the increase of squalene doses (Table V), and these results are similar to those of the previous study. That is, Ohkuma *et al.*[32] reported that number of splenic plaque forming cells to SRBC was enhanced by squalene treatment in the healthy and the tumor-bearing mice. In view of their report, it is thought that squalene may augment the humoral immune responses by both the activation of B cells and immunoglobulins synthesis.

Arthus reactions were slightly enhanced in squalene treatment as shown in Table VI. These results indicate that squalene slightly enhanced B-cell dependent immunoglobulin G as well as complement.

Circulating leukocyte count was significantly increased in the squalene treatment, and the results were dose-dependent as shown in Table VII. These results seem to suggest that the increase of squalene doses may augment circulating leukocyte counts by the enhancement of lymphocyte activity.

While the squalene plus cyclophosphamide-treated groups decreased humoral immune responses in comparison with those of the non-treated control group, but significantly enhanced humoral immune responses in comparison with those of the cyclophosphamide (5 mg/kg)-treated control group. And these results are similar to those of the previous studies, that is, several papers[33-35] reported that humoral immune respsonses were decreased by cyclophosphamide treatment. These results seem to suggest that the suppressive effect of cyclophosphamide on humoral immune responses was significantly prevented by squalene treatment.

In conclusion, squalene treatment resulted in enhancement of humoral immunity.

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