INTENSIFICATION OF HOST'S IMMUNITY BY SQUALENE IN SARCOMA 180 BEARING ICR MICE

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Effect of highly purified squalene (HP-SQ) obtained from liver of a shark, *Centrophorus atromarginative* GARMAN, on antitumor activity and host's immune response was studied in sarcoma 180 bearing female ICR mice. HP-SQ was administered intraperitoneally (*i.p.*) to solid tumor-bearing or healthy mice at the dose of 0.1 ml/mouse/d for consecutive 10 d. The administration of HP-SQ exerted the host's resistance against challenging 5×10^4 cells of ascites sarcoma 180, and it resulted in marked prolongation of survival of the mice (ILS; 110%). Function of the reticuloendothelial system estimated by a carbon clearance test was enhanced by *i.p.* administration of HP-SQ in both the healthy and the tumor-bearing mice. Number of IgM antibody forming cells against sheep red blood cells (SRBC) in the spleen was counted 4 d after intravenous injection of 2×10^7 cells of SRBC. The number of the IgM antibody forming cell increased by HP-SQ administration in the tumor-bearing and the healthy mice. Delayed hypersensitivity reaction against SRBC measuring swelling of the foot pads of mice was also stimulated by the *i.p.* administration of the material.

Keywords — squalene; antitumor activity; sarcoma 180; host's immunity; carbon clearance activity; IgM; delayed hypersensitivity

Mononuclear phagocytes (macrophages) play various important roles in the host's resistance against foreign antigens.¹⁾ Antigen-specific and non-specific phagocytosis of alien substances is one of the important functions of macrophages,²⁾ and transfer of an antigen-specific information to the lymphocytes is an evolutionary function of the mononuclear cells.³⁾ Macrophage activating substances, such as bacterial preparations,⁴⁾ polysaccharides,⁵⁾ and endotoxin⁶⁾ were illustrated to exhibit antitumor activity against transplantable tumors. J.H.Heller *et al.* reported that partially purified lipid extracted from a shark liver (about 20% of the wet weight) exhibited stimulation of the reticuloendothelial system.⁷⁾ And this oil stimulated host's resistance against bacterial infection as well as transplantable tumor challenge by the combined use of the respective antigen.^{7,8)} Taking into account of physico-chemical properties and the preparation procedure of the oil, it

is assumed that the shark liver oil must highly contain squalene.

Recently, T.Ikekawa *et al.* reported antitumor activity of highly purified squalene (HP-SQ) in murine transplantable tumor systems.⁹⁾ This paper concerns with the antitumor effect and the immuno-stimulating effect of the HP-SQ in sarcoma 180 bearing female ICR mice. HP-SQ (squalene content: 99.8%) obtained from liver of a shark, *Centrophorus atromarginative* GARMAN, was supplied by Nisei Marine Corp. (Tokyo).

Effect of the HP-SQ on the host's resistance against challenged ascites sarcoma 180 was shown in Table I. Mice of group 1 and 3 were subcutaneously (*s.c.*) transplanted with 5×10^6 cells of ascites sarcoma 180 on day -14. Mice of group 1 and 2 were administered 0.1 ml of HP-SQ intraperitoneally (*i.p.*) 10 times on day -12—-8 and -6—-2. On day 0, all of the

mice of group 1–4 were *i.p.* challenged with 5×10^4 cells of ascites sarcoma 180, and the survival days of each mouse were estimated. *i.p.* Administration of HP-SQ exerted host's resistance against challenged ascites sarcoma 180 (group 1 and 2), and the marked antitumor effect of HP-SQ was recognized in the mice bearing solid tumors (group 1). On the other hand, T.Ikekawa, *et al.* reported that this material did not exhibit a direct cytotoxic activity on the tumor cells both *in vitro* and *in vivo*.⁹⁾

In order to examine the effect of HP-SQ on the host's immunity, function of the reticuloendothelial system (RES),¹⁰⁾ delayed hypersensitivity (DTH)¹¹⁾ and IgM antibody production¹²⁾ were studied. Each experiment consisted of 4 groups of mice (5 mice in each group), *i.e.* 1) *s.c.* tumor bearing + squalene, 2) squalene, 3) *s.c.* tumor bearing, 4) control. Mice bearing *s.c.* tumor were created by transplanting 5×10^6 cells of sarcoma 180 on day -14. Squalene (HP-SQ, 0.1 ml) was *i.p.* administered daily for consecutive 10 d, starting on day -13 and ending on day -4. On day 0, immunologic status of the animals was investigated.

Carbon clearance activity¹⁰⁾ of the mice, injected with 0.01ml/g body weight of Pelikan C11/1431 ink (30mg/ml) diluted in 1% gelatin containing saline into the tail vein, was examined as a RES test.¹⁰⁾ Optical density (660nm) of hemolysed venous blood 1 and 15 min after the carbon injection was measured. The phagocytic index, K , was calculated by the following formula:

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

C_1 and C_2 are the carbon levels at the time T_1 and T_2 , respectively. As shown in Fig. 1, venous carbon levels 15 min after the carbon injection were significantly lowered by HP-SQ administration in *s.c.* tumor bearing and control mice comparing to non-treated control mice ($p < 0.01$). Though the mice injected zymozan *i.p.* twice at the dose of 100mg/kg (Sigma, U.S.A.) on day -4 and -2 showed the highest carbon clearance activity in this experiment, *i.p.*

administration of HP-SQ augmented macrophage's phagocytic activity of both tumor-bearing and the healthy hosts.

Fig. 2 shows the result on DTH reaction of the mice against sheep red blood cells (SRBC).¹¹⁾ Mice were sensitized *i.p.* to SRBC (1×10^8 cells/mouse) 4 d before the test. Swelling of the foot pad induced by *s.c.* injection of 20 μ l of 20% SRBC in saline was measured 24 h after the challenge of the antigen. The healthy mice receiving HP-SQ showed a high DTH reaction, but the tumor-bearing mice administered with the

FIG. 1. Carbon Clearance Activity of Mice (see test) ---△---: healthy mice ($K=0.007$), ---■---: tumor-bearing mice ($K=0.007$), —●—: tumor-bearing mice administered with squalene ($K=0.009$), —□—: healthy mice administered with squalene ($K=0.006$), ---○---: healthy mice administered with zymozan ($K=0.007$).

Bars: SE, a) $p < 0.01$ (against healthy mice).

TABLE I. *Antitumor Effect of Highly Purified Squalene*

Experimental group	Treatment	Mean survival days ($\pm$ SE)	Increase in life span (%)	Body weight gain (g, $\pm$ SE) ^{a)}	No. of 60 d survivors
					No. in group
1	Tumor-bearing + Squalene	42.9 $\pm$ 3.9 ^{b)}	110.4	4.0 $\pm$ 0.3	1/8
2	Squalene	39.1 $\pm$ 4.3 ^{c)}	92.0	3.4 $\pm$ 0.5	1/8
3	Tumor-bearing	29.6 $\pm$ 4.5	45.4	3.5 $\pm$ 0.7	0/8
4	Control	20.4 $\pm$ 1.5	0	2.6 $\pm$ 0.6	0/8

All of the mice were *i.p.* challenged 5×10^4 cells of ascites sarcoma 180 on day 0, and the survival days of each mouse were estimated. Mice of group 1 and 3 were bearing *s.c.* tumor by transplanting 5×10^6 cells of sarcoma 180 on day -14. Mice of group 1 and 2 were administered with 0.1 ml of HP-SQ *i.p.* 10 times on day -12--8 and -6--2.

a) difference in body weight of mice between on day -14 and on day 0.

b) significantly different from control $p < 0.001$, and c) $p < 0.01$.

FIG. 2. *Delayed Hypersensitivity Reaction against Sheep Red Blood Cells*

a) significantly different from healthy control, $p < 0.05$.

material exhibited a relatively weak reaction. This result suggests that HP-SQ intensified DTH inducing cells activity, *i.e.* T-lymphocytes' function, of the healthy and the tumor-bearing hosts.

FIG. 3. *Number of IgM Antibody Forming Cells in Spleens*

White columns indicate the number of plaque forming cells/ 1.6×10^5 spleen cells and shaded columns represent the number of plaque forming cells/spleen $\times 10^4$. a) Significantly different from healthy control ($p < 0.05$), and from tumor-bearing mice ($p < 0.01$).

Effect of HP-SQ on a humoral immune response was illustrated in Fig. 3. Cunningham and Szenberg method was applied to detect plaque-forming cells (PFC).¹²⁾ Number of PFC in the spleens of the experimental animals was counted 4 days after the sensitization of the intravenous injection of 2×10^7 SRBC. The PFC are reported to be anti-SRBC IgM antibody producing cells.¹³⁾ Competence of anti-SRBC IgM antibody production was suppressed in the tumor-bearing hosts, but was enhanced by HP-SQ administration in the healthy and the tumor-bearing mice.

In this study, we demonstrated augmentation of immunologic reactions of tumor-bearing animals by *i.p.* administration of the HP-SQ. And this *i.p.* administration of HP-SQ did not reduce body weight of the mice in comparison with the control (Table I), and the mice administered with HP-SQ survived longer than non-treated mice after *i.p.* challenging of ascites sarcoma 180. Moreover, it is reported by T.Ikekawa *et al.* that oral administration of HP-SQ exerted anti-tumor activity.⁹⁾ In cancer immunotherapy, augmentation of the tumor-specific as well as non-specific immunity is critical and important.¹⁴⁾ Biological functions of the new immunostimulating material, HP-SQ have to be clarified: its immunologic feature, and its metabolism.

In this study, statistical analysis was made by Student's t-test.

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