A Biological Study on Hot-Water Extract from Delipidated *Mycobacterium bovis* Strain BCG

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— The biological activity of a hot-water extract from delipidated BCG, designated as HSA (Hot-water Soluble Adjuvant), was investigated. The HSA did not induce hyperreactivity to bacterial endotoxin. The hot-water extract from which nucleic acids had been removed by streptomycin (SM-HSA) was found to enhance the delayed-type hypersensitivity as evidenced by the results of footpad reaction of mice. The HSA and SM-HSA could be injected to mice by an intraperitoneal route for 20 consecutive days without undesirable side effects. A comparative study was made on the effects of HSA in relation to the duration and doses of treatment with HSA using the ddI mice inoculated with Sarcoma-180. The most remarkable effect was observed when 0.25 mg of HSA had been injected for 20 consecutive days. Also SM-HSA was found to exert antitumor activity when applied in the same manner as above. These results suggest that the presence of nucleic acids is not related to the biological and antitumor activities of the hot-water extract. ——— hydro-soluble adjuvant; BCG; antitumor activity

A hot-water extract from delipidated *Mycobacterium bovis* strain BCG was found to be adjuvant-active and was designated as HSA (Hot-water Soluble Adjuvant; Sato et al. 1976). It is composed of neutral sugars (mainly mannose and glucose), amino acids, and approximately 10% of nucleic acids. Its effectiveness in the treatment of mice inoculated intraperitoneally with Sarcoma-180 cells and Ehrlich carcinoma cells has also been demonstrated (Sato et al. 1978). The chemical composition of the hot-water extract differs markedly from that of other water soluble adjuvants which have so far been extracted from mycobacterial cell walls (Adam et al. 1972, 1973; Hiu 1972; Migliore-Samour and Jolles 1972, 1973). When evaluated by the results of chemical analyses, it is likely that HSA is derived from cytoplasm rather than from cell wall (Sato et al. 1976). On the other hand, nucleic acids have been reported to be adjuvant-active and to exert antitumor activity (Millman et al. 1974, 1976; Casavant and Youmans 1975). Thus, it is necessary to exclude the possibility that the biological activities of the hot-water

extract are contributed by nucleic acids. In this study, the biological activity of the hot-water extract was investigated after nucleic acids had been removed by streptomycin sulfate.

MATERIALS AND METHODS

HSA (Hot-water Soluble Adjuvant) was prepared as described previously (Sato et al. 1976).

Treatment with streptomycin sulfate of HSA for the removal of nucleic acids

One g of HSA, dissolved in 300 ml of distilled water, received the dropwise addition of 30 ml of a 10% solution of streptomycin sulfate (SM). The opalescent solution thus obtained was centrifuged at $10,000 \times g$ for 30 min at 4°C and 1/10 volume of a 10% SM-solution was added to the supernatant. After centrifugation at $10,000 \times g$ for 30 min at 4°C , the clear supernatant was dialyzed against running tap water to remove SM and the retentate was lyophilized. Quantitative determination of nucleic acids was carried out according to the method of Schmidt and Tannhauser (1945), and Schneider (1946).

Biological activities

Survival rate of mice after injection of endotoxin. Three groups of 30 male ddI mice weighing 20 to 22 g were injected intravenously with saline, 0.3 mg of BCG and 0.3 mg of HSA, respectively. Fourteen days after, they were challenged by the same route with various doses of endotoxin extracted from *Salmonella enteritidis* by the phenol-water procedure (Westphal et al. 1952). The number of dead mice within 72 hr after challenge was recorded.

Footpad reaction. Male ddI mice were injected intraperitoneally with 0.25 mg of HSA and streptomycin-treated HSA (hereafter SM-HSA) 14 days and 4 days before sensitization respectively. As an sensitizing antigen, 10^8 sheep red blood cells were injected intraperitoneally. After 4 days, the sensitized mice received an intradermal injection of 10^8 sheep erythrocytes on the right footpad. The thickness of the right footpad was compared with that of the non-injected (left) footpad after 24 hr.

Gross findings of the liver and spleen

1) Four groups of 20 ddI mice each were injected intravenously with 0.25 ml of saline containing 0.25 mg of HSA, 0.5 mg of HSA, 0.25 mg of SM-HSA and 0.5 mg of SM-HSA, respectively, at days 1 and 8. At day 22 the mice were killed by cervical dislocation and the liver and spleen were weighed.

2) Eighty ddI mice consisting of 4 groups of 20 mice each, received intraperitoneal injections of 0.25 mg of HSA or SM-HSA for 10 and 20 consecutive days respectively. Fourteen days after the final injection the mice were sacrificed and the weight of the liver and spleen was determined.

Antitumor effect on Sarcoma-180 of HSA and SM-HSA

Age-matched, adult male ddI mice were obtained from the animal center of Tohoku University. Various doses of HSA and SM-HSA were taken up in 0.25 ml of saline and were injected intraperitoneally. Sarcoma-180 was maintained in ascites by weekly inoculations. After evaluation of the viability by the dye exclusion test, the number of tumor cells was adjusted with saline as required immediately before inoculation.

Experiment 1 (Effectiveness of regimens in relation to the duration of treatment). Eighty mice were divided into 4 equal groups of 20 mice. They were inoculated with 4×10^6 tumor cells. From 1 day after inoculation, 3 groups were given 0.25 mg of HSA for 7, 14 and 21 consecutive days respectively. The fourth group served as a control.

Experiment 2. (Effectiveness of regimens in relation to the doses of HSA). Five groups of mice consisting each of 20 mice received intraperitoneal injections of 3×10^6 tumor cells. From 1 day after inoculation of tumor cells, 4 groups were injected with 0.005, 0.01, 0.10 and 0.25 mg of HSA, respectively, for 20 consecutive days. The fifth group was given saline alone. The survival days were compared among the above four experimental groups and a control group.

Experiment 3. One hundred mice were inoculated with 3×10^5 tumor cells and were divided into 5 equal groups of 20 mice. Two of the 5 groups were injected with 0.25 mg of HSA and 0.25 mg of SM-HSA, respectively, for 20 consecutive days from day 1 after inoculation of tumor cells. The other 2 groups received 10 injections of 0.5 mg of HSA and 0.5 mg of SM-HSA, respectively, every other day from day 1 after inoculation of tumor cells. The survival time in days in the above 4 groups were compared with that of the control group which had received saline alone.

Experiment 4. Ninety mice, divided into 3 equal groups of 30 mice, were inoculated with 3×10^5 tumor cells. The first group received injections of 0.25 mg of HSA from day 7 after inoculation of tumor cells for 20 consecutive days. The second group was treated with injections of 0.25 mg of SM-HSA in the same manner as in the first group. The third group that had been injected with saline alone served as a control. The number of surviving mice was compared among three groups.

Statistical analysis

The Student *t*-test was used in all statistical evaluations.

RESULTS

Treatment with streptomycin sulfate of HSA

After treatment with SM, the rate of recovery of SM-HSA was 85 to 90%. These results substantiate the data of the chemical analyses which showed the presence of 10 to 15% of nucleic acids in HSA. The absence of nucleic acids in SM-HSA was confirmed by the disappearance of absorbance at 260 nm. In addition it was found that SM-HSA was free from nucleic acids also by quantitative analysis.

Footpad reaction

As shown in Table 1, an increase of the thickness of the footpad was observed not only in mice which had been given HSA but also in mice which had received SM-HSA ($p < 0.001$). Thus, also SM-HSA which is devoid of nucleic acid seems to have enhanced the delayed-type hypersensitivity to sheep red blood cells.

Survival rate of mice after injection of endotoxin

Table 2 summarizes the survival rate of mice after injection of endotoxin from *Salmonella enteritidis* after treatment with BCG and HSA, respectively. Five of 10 mice in the group which had received an intravenous injection of BCG were dead 3 days after administration of 0.015 mg of endotoxin. However, there was no death in the group which had been treated by HSA.

Effect on weight of the liver and spleen

Tables 3 and 4 show the weight of the liver and the spleen in the mice treated

TABLE 1. *Footpad reaction*

Injected with	Day	Increase in footpad thickness (10^{-1} mm) mean $\pm$ s.d.
Saline	-14	0.8 ± 0.7
Saline	-4	0.9 ± 0.7
HSA	-14	$1.7 \pm 0.8^*$
HSA	-4	$3.2 \pm 1.1^*$
SM-HSA	-14	$2.8 \pm 1.0^*$
SM-HSA	-4	$2.0 \pm 0.6^*$
BCG	-14	$1.9 \pm 1.5^*$
BCG	-4	$3.5 \pm 1.3^*$

* $p < 0.001$

Male ddI mice, 10 animals per group, were injected with 0.25 mg of HSA, SM-HSA and BCG intraperitoneally 14 and 4 days respectively prior to the intraperitoneal injection of 10^8 of sheep red blood cells (SRBC). After 4 days (on day 4), 10^8 SRBC were injected into the right hind footpad and the difference in thickness of footpad was recorded 24 hr later.

TABLE 2. *Effect of BCG and HSA on survival rate of mice after injection of endotoxin*

Treatment	Endotoxin (μ g)	Dead/Total
Untreated control	100	0/10
	200	2/10
	400	7/10
BCG 0.3 mg	1.5	0/10
	5	0/10
	15	5/10
HSA 0.3 mg	50	0/10
	100	0/10
	200	0/10

Mice were given 0.3 mg of BCG or 0.3 mg of HSA intraperitoneally. Fourteen days later, varying doses of endotoxin were injected by the same route. The number of deaths within 72 hr was compared.

with intravenous and intraperitoneal injections of HSA and SM-HSA. No remarkable increase in weight of either the liver or the spleen was observed in any group.

Antitumor activity

Experiment 1. Fig. 1 demonstrates the results of the experiment designed to compare the effects of various regimens of HSA in relation to the duration of treatment. Of the 3 groups which had received HSA the survival time was prolonged most remarkably in the group that had been injected for 21 consecutive days, as shown by the fact that the survival rate of this group at 60th day was 50%.

TABLE 3. *Effect of HSA and SM-HSA on the weight of liver and spleen of mice*

Treatment	Weight	
	Liver (mg) mean $\pm$ s.d.	Spleen (mg) mean $\pm$ s.d.
Saline	2004 $\pm$ 244	164 $\pm$ 32
HSA, 0.25 mg	1920 $\pm$ 246	146 $\pm$ 36
HSA, 0.50 mg	2056 $\pm$ 276	168 $\pm$ 75
SM-HSA, 0.25 mg	2137 $\pm$ 272	159 $\pm$ 55
SM-HSA, 0.50 mg	2135 $\pm$ 224	161 $\pm$ 58

Mice were injected intravenously with 0.25 ml of saline containing 0.25 mg and 0.5 mg of HSA at days 1 and 8, respectively, SM-HSA was injected in a similar manner. At day 22, mice were sacrificed and the weights of the liver and spleen were determined.

TABLE 4. *Effect of HSA and SM-HSA on the weights of liver and spleen in mice*

Treatment	Duration (day)	Weight (mg)	
		Liver mean $\pm$ s.d.	Spleen mean $\pm$ s.d.
Saline	1-20	1442 $\pm$ 135	112 $\pm$ 52
HSA	1-20	1502 $\pm$ 140	132 $\pm$ 22
HSA	1-10	1196 $\pm$ 64	107 $\pm$ 15
SM-HSA	1-20	1515 $\pm$ 128	135 $\pm$ 18
SM-HSA	1-10	1197 $\pm$ 104	127 $\pm$ 12

Mice received intraperitoneal injections of 0.25 mg of HSA and SM-HSA respectively for 10 or 20 consecutive days. Fourteen days after the final injection, mice were sacrificed and the weights of the liver and spleen were compared.

Fig. 1. Comparison of the effect of HSA on survival rate of mice in relation to the length of treatment.

Group 1 was injected with 0.25 mg of HSA for 7 consecutive days intraperitoneally from day 1 after inoculation of 4×10^5 tumor cells. Group 2 was injected with 0.25 mg of HSA for 14 consecutive days and Group 3 was injected with 0.25 mg of HSA for 21 consecutive days respectively in the same manner as in Group 1.

Experiment 2. As shown in Fig. 2, the survival time was most markedly prolonged in the group that had been given 0.25 mg of HSA. At 60th day after inoculation of tumor cells, the survival rate of the mice that had been given 0.25 mg of HSA was 55%, whereas that of the groups that had received 0.10, 0.01, and 0.005 mg of HSA were 40%, 30% and 20%, respectively.

Fig. 2. Comparison of doses of HSA.

Group 1 received 0.005 mg of HSA intraperitoneally for 20 consecutive days from day 1 after inoculation of 3×10^6 cells of Sarcoma-180. Group 2, Group 3 and Group 4 received 0.01, 0.1 and 0.25 mg of HSA respectively in the same manner as in Group 1.

Fig. 3. Antitumor activity of HSA and SM-HSA.

Group 1 was given 0.25 mg of HSA intraperitoneally for 20 consecutive days from day 1 after the inoculation of 3×10^5 cells of Sarcoma-180. Group 2 was given 0.25 mg of SM-HSA in the same manner as in Group 1. Group 3 was given 0.25 mg of HSA intraperitoneally 10 times every other day from day 1 after inoculation of tumor cells. Group 4 was given 0.25 mg of SM-HSA in the same manner as in Group 3.

Experiment 3. As demonstrated in Fig. 3, both HSA and SM-HSA were found to be effective. At 50th day after inoculation of tumor cells, the survival rates of

the groups that had been injected with 0.25 mg of SM-HSA and 0.25 mg of HSA for 20 consecutive days were 90% and 65%, respectively. In the groups that had been given 0.5 mg of HSA 10 times every other day, the survival rates were 45% and 30% at 50th day.

Experiment 4. As illustrated in Fig. 4, even when the treatment was initiated 7 days after inoculation of tumor cells, the antitumor effect was found in the groups which had been injected with HSA and SM-HSA, respectively.

Fig. 4. Antitumor activity of HSA and SM-HSA.

Group 1 was treated by intraperitoneal injections of 0.25 mg of HSA for 20 consecutive days from day 7 after inoculation of 3×10^5 tumor cells. Group 2 was treated with 0.25 mg of SM-HSA in the same manner as in Group 1.

DISCUSSION

A hot-water extract obtained from defatted BCG has been found to be adjuvant-active and to have antitumor activity. Since this substance contained approximately 10% of nucleic acids, it was necessary to remove the nucleic acids to find out whether the biological activity of this extract was related to the presence of nucleic acids. Enzymes have often been employed for the elimination of nucleic acids. However, it is difficult to remove the enzymes completely from the reaction mixture. Thus, in this study streptomycin sulfate (SM) was used for the removal of nucleic acids.

The results of the footpad reaction seem to indicate that delayed-type hypersensitivity can be induced also by SM-HSA which is devoid of nucleic acids. Whether the injection of SM-HSA results in an increase in titer of humoral antibody is now under investigation.

Since the reports of Halpern et al. (1958) and Suter et al. (1958), it has widely been known that mice inoculated intravenously with BCG become hyperreactive to bacterial endotoxin. This increase of sensitivity to endotoxin has been reported to be related to the formation of granulomas in various organs. When HSA was given to mice intravenously, hyperreactivity to endotoxin was not observed. This result was in agreement with the results obtained with other water-soluble

adjuvants from mycobacterial preparations (Chedid et al. 1972; Adam et al. 1973).

Neither the liver nor the spleen was enlarged in the mice which had been injected intravenously with HSA or SM-HSA. The mice could tolerate the intraperitoneal injections of these substances even when administered for 20 consecutive days.

There have been only a few reports concerning the antitumor activities of water-soluble substances of mycobacterial origin (Hiu 1972; Mathe et al. 1973, 1975). Our previous publication reported that HSA was effective on Sarcoma-180 and Ehrlich carcinoma when injected intraperitoneally for 7 consecutive days. However, the doses and duration for the most satisfactory treatment with HSA had not yet been established. The HSA in the present experiment caused no undesirable side effects in the mice which had received intraperitoneal injections for 20 consecutive days. Hence in this study the antitumor effect of HSA was compared among three groups which had been treated for 7, 14 and 21 consecutive days, respectively. As a result it was found that survival time was prolonged most remarkably in the 21-day group.

In the experiment in which varying amounts of HSA were given for 20 consecutive days, the most remarkable increase of survival time was observed in the group that had received 0.25 mg of HSA. Thus in the experiment designed to evaluate the antitumor effect of SM-HSA, mice received 0.25 mg of SM-HSA for 20 consecutive days. SM-HSA was found to be as effective as HSA when treatment had been initiated 1 day after inoculation of tumor cells. Also in the experiment in which treatment was begun at 7th post-inoculation day when ascites had appeared already, the survival time became longer in both HSA and SM-HSA groups.

The results of the experiments described above seem to indicate that nucleic acids are not essential for the manifestation of the biological activity of the hot-water extract. Further fractionation of SM-HSA is now being carried out in order to find out the principal components necessary for biological activities.

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