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Biological Activities of Mouse Haptoglobin Elicited by Administration of an Antitumor Polysaccharide, PSK*

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The concentration of mouse haptoglobin in serum was increased by administration of an antitumor polysaccharide, PSK. The administration of the purified mouse haptoglobin inhibited the growth of Sarcoma-180 cells implanted in ICR mice. Furthermore, this glycoprotein enhanced macrophage activities *in vitro*, as judged from the cytostatic and cytolytic activities, glucose consumption, O_2-production, and interleukin-1 production of macrophages. In addition, mouse haptoglobin enhanced the cytolytic effect of cytotoxic T-lymphocytes. These results suggested that haptoglobin has a role in restoring or enhancing the resistance of the host against tumors.

Maeda *et al.* [1] reported marked increases in three electrophoretically separable serum protein components in the regions of α- and β-globulins, designated as LA, LB and LC, soon after the administration to mice of a potent anti-tumor polysaccharide, β(1-3)-glucan, isolated from an edible mushroom, *Lentinus edodes*. This phenomenon has been observed also on the administration of various anti-tumor polysaccharides, lipopolysaccharides and bacterial cell walls [2]. PSK employed in this study is one of these anti-tumor polysaccharides which was isolated from a fungus belonging to the Basidomycetes, *Coriolus versicolor*, and is a glucan having a molecular weight of 9.4×10^4 [3].

Abbreviations: FCS, fetal calf serum; LPS, lipopolysaccharide; CTL, cytotoxic T-lymphocytes; IL-1, interleukin 1.

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We have previously reported that LA, LB and LC are mouse ceruloplasmin [4], hemopexin [5] and haptoglobin [6], respectively. We have also reported that mouse hemopexin inhibits the growth of Sarcoma-180 cells *in vivo* [7] and enhances the tumor cell killing ability of activated macrophages *in vitro* [8]. Human ceruloplasmin was also found to inhibit the growth of Sarcoma-180 cells *in vivo* [9]. Furthermore, studies on the mechanism of the antitumor activity of ceruloplasmin revealed that the glycoprotein has a neutralizing effect on various toxohormone-like activities of the basic protein isolated from Ehrlich carcinoma cells [10], and a restorative effect on the decrease in both delayed hypersensitivity and helper T-cell activity in Sarcoma-180 bearing mice. Moreover, it enhances the activity of alloreactive cytotoxic cells *in vitro* [10].

In the present work, we examined the effect of mouse haptoglobin on the growth of Sarcoma-180 cells implanted in ICR mice and some of its biological activities against macrophages and cytotoxic T-lymphocytes in comparison with mouse hemopexin, which was found to have an enhancing effect on the activity of tumoricidal macrophages, mainly through the enhancement of the intimate binding between activated macrophages and tumor cells [8].

Materials and Methods

Mice

Female ICR-JCL mice (CLEA, Japan Inc., Tokyo, Japan), 7-10 weeks old, and female DBA/2 and C3H/HeN mice (Charles River Japan, Kanagawa, Japan), 7-10 weeks old, were used in this study.

Media and Reagents

RPMI 1640 and fetal calf serum (FCS) were purchased from Grand Island Biological Co. (Grand Island, NY, USA), and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) from Wako Pure Chemical Co. (Tokyo, Japan). The RPMI medium used in this study consisted of RPMI 1640 supplemented with 25 mM HEPES-NaOH (pH 7.2), 4 mM glutamine, 1 mM pyruvate, 60 μg/ml kanamycin (Meiji Seika Co., Tokyo, Japan), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 5-10% FCS. Eagles minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) contained 25 mM HEPES. Proteose peptone, purchased from Difco Laboratories (Detroit, MI, USA), was used as a 10% w/v solution. Lipopolysaccharide (LPS) of *Salmonella enteritidis* (No. L-3130, phenol extract) was obtained from Sigma Chemicals (St. Louis, MO, USA), phytohemagglutinin (PHA) from Burroughs-Wellcome (Research Triangle Park, NC, USA), and picibanil (OK-432), an antitumor Streptococcus preparation [11], was supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). PSK was kindly supplied by Kureha Chemical Co. (Tokyo, Japan). Five percent PSK in saline was prepared for injection. $Na_2{}^{51}CrO_4$ (specific activity, 350 mCi/mg) was purchased from New England Nuclear (Boston, MA, USA) and ^{3}H-thymidine (specific activity, 9.21 Ci/mmol) from the Radiochemical Centre (Amersham, UK).

Cells

Sarcoma-180, a transplantable sarcoma, was passaged weekly in the peritoneal cavities of ICR mice, and X-5563, a transplantable plasma cytoma, was passaged in those of C3H/HeN mice. The C3H/HeN fibrosarcoma line, C3MC2, was maintained *in vitro* in RPMI medium supplemented with 10% FCS. Peritoneal exudate macrophages were isolated from C3H/HeN mice four days after intraperitoneal injection of 2.0 ml of bacto-thioglycolate medium (2.4%, w/v; TGC-Mϕ), 1.5 ml of proteose-peptone solution (PP-Mϕ, 1.5 ml of proteose-peptone solution (PP-Mϕ) or 0.2 ml of OK-432 (10 KE/ml; OK-Mϕ). One KE (Klinishe Einheit) of OK-432 corresponds to 0.1 mg dried streptococci. Cells were cultured on 24 or 96 well plates (Falcon Plastics, Oxnard, CA, USA) in RPMI medium supplemented with 10% FCS. After 2 h at 37°C, the cultures were washed twice to remove non-adherent cells.

Purification of Mouse Haptoglobin and Hemopexin

The purification of mouse haptoglobin and hemopexin was carried out by the methods described previously [6, 7]. The glycoproteins used in this study were confirmed to be homogeneous by polyacrylamide gradient gel electrophoresis [7].

Preparation of Serum from Mice Injected with PSK (PSK-Serum)

Each of three ICR-JCL mice, injected i.p. with PSK, was bled from the heart and the blood samples were allowed to clot at room temperature for 1-2 h. The serum was obtained by centrifugation at 2 000 rpm for 5 min.

Quantitative Estimation of Haptoglobin

For quantitative estimation of haptoglobin, the peroxidase activity of a sample was measured after addition of a standard amount of human methemoglobin by the method of Owen *et al.* [12]. Purified mouse haptoglobin was used as a standard in this assay. Protein was determined by the method of Lowry *et al.* [13] with bovine serum albumin as a standard.

Evaluation of the Effect of Tumor Cells in Vivo

Washed Sarcoma-180 cells (2×10^6 cells) were implanted subcutaneously in the right groins of ICR mice (six mice/group) and 0.2 ml of various concentrations of the test sample was intravenously injected daily for five days, starting from 24 h after tumor implantation. Tumor weights were determined on day 26 after inoculation.

Tumoricidal Activity of Macrophages

C3MC2 was labeled with ^{3}H-thymidine, as described by Ruco *et al.* [14]. Briefly, OK-Mϕ in a 96 well plate (1×10^5 cells/well) were cultured with labeled tumor cells (1×10^4 cells/well) in RPMI 1640 medium supplemented with 10% FCS in the presence of a 1/10 volume of a sample. The total volume was 0.2 ml per well. After 48 h at 37°C under an atmosphere of 5% CO_2 and 95% air, the plate was centrifuged. The radioactivity released

from dead target cells was counted in 0.1 ml of each supernatant with the use of Triton X-100-toluene scintillator. The lysis of target cells was expressed as the percentage specific release of radioactivity:

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Spontaneous release was determined without macrophages and total release was determined with the addition of 10% sodium dodecyl sulfate (SDS) instead of a sample.

Assay for Glucose Consumption by Macrophages

PP-Mϕ in a 96-well plate (2.5×10^5 cells/well) were cultured in RPMI 1640 medium supplemented with 15% FCS, in the presence of a 1/10 volume of a sample. The total volume was 0.1 ml per well. After 24 h at 37°C under an atmosphere of 5% CO_2 and 95% air, 10 μl of each supernatant were harvested, and the concentration of glucose was measured with a "Glucose-B-test-Wako" kit (Wako Pure Chemical Co., Tokyo, Japan). Glucose consumption was expressed as a percent ratio:

$$\% \text{ glucose consumption} = \frac{[\text{glucose}] \text{ in the medium} - [\text{glucose}] \text{ in the test supernatant}}{[\text{glucose}] \text{ in the medium}} \times 100$$

where [glucose] is the glucose concentration

Assay for Superoxide Production by Macrophages

PP-Mϕ in a 96 well plate (1×10^4/well) were cultured in RPMI 1640 medium supplemented with 5% FCS in the presence of a 1/10 volume of a sample. The total volume was 50 μl per well. After 3 days at 37°C under an atmosphere of 5% CO_2, 10 μl of nitro-tetrazolium blue (NBT) solution (1.1% w/v, in double distilled water) was added to each well. After 1 h culture, NBT positive cells were counted (cells/area).

$$\% \text{ NBT-positive cells} = \frac{\text{NBT-positive cells/area}}{\text{total cells/area}} \times 100$$

Assay for Interleukin-1 (IL-1) Production by Macrophages

IL-1 supernatants were prepared essentially according to Unanue and Kiely [15]. Briefly, PP-Mϕ monolayers in a 24 well plate (2×10^6 cells/well) were cultured with a 1/10 volume of a sample in RPMI 1640 medium supplemented with 5% FCS in the presence or absence of LPS (20 μg/ml). The total volume was 1.0 ml per well. After 48 h at 37°C, under an atmosphere of 5% CO_2 and 95% air, the cell free supernatants were extensively dialyzed against RPMI 1640 and then used as IL-1 supernatants. The same medium incubated without cells was used as the control supernatant. IL-1 activity was assayed according to the thymocyte proliferation assay method reported by Mizel *et al.* [16]. Briefly, a suspension of thymocytes from C3H/HeN mice was cultured for 72 h at 1.5×10^6 cells/well in flat bottom wells (Costar 96 well, 3596) with a 1/2 volume of filtered IL-1 supernatants or the control supernatant in RPMI 1640 medium supplemented with 20% FCS in the presence of PHA (1 μg/ml). The total volume was 0.2 ml per well. ^{3}H-Thymidine (0.5 μCi/well) was added for the final 4 h, the cells were harvested with a semiautomatic multiple cell harvester and radioactivity was determined with a liquid scintillation counter.

Induction of Cytotoxic T-Lymphocytes (CTL) and Assay for CTL Activity

Spleen cells from DBA/2 mice bearing 2 weeks-old tumors (2×10^6 cells, s.c., X-5563) were used as responder cells. X-5563 cells treated with mitomycin C (100 μg/ml) for 1 h at 37°C were used as stimulator cells. Responding spleen cells in a 24-well plate (5×10^6/well) were cultured with stimulator cells (1×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FCS, in the presence of a 1/20 volume of sample. The total volume was 2.0 ml per well. After 5 days at 37°C under an atmosphere of 5% CO_2 and 95% air, the cells were used as effector cells. CTL activity was assayed using ^{51}Cr-labeled X-5563 cells as target cells, according to the method of Brunner *et al.* [17]. Briefly, effector cells in a 96 well plate were cultured with labeled tumor cells (5×10^3 cells/well) in RPMI 1640 medium supplemented with 5% FCS. The total volume was 0.2 ml per well and the effector:target ratio was 7:1, 14:1 or 28:1. After 4 h at 37°C under an atmosphere of 5% CO_2 and 95% air, the plate was centrifuged. The radioactivity released from dead target cells was counted in 0.1 ml of each supernatant with an auto-well gamma counter, ARC-500 (Aloka). The lysis of target cells was expressed as the percentage specific release of radioactivity, calculated as described above. Spontaneous release was determined without effector cells and total release was taken as the radioactivity released from target cells dissolved in NaOH solution.

Results

Concentration of Haptoglobin in Mouse Serum

The change on standing of the total quantity of haptoglobin in mouse serum after administration of PSK was determined as peroxidase activity, with purified mouse haptoglobin as a standard. As shown in Fig. 1, the concentration of total haptoglobin was 2.3 mg/ml in the case of the control. The concentration increased remarkably, up to 14.5 mg/ml (day 2), after injection with PSK. However, injection of isolichenan (an α(1-3)- and

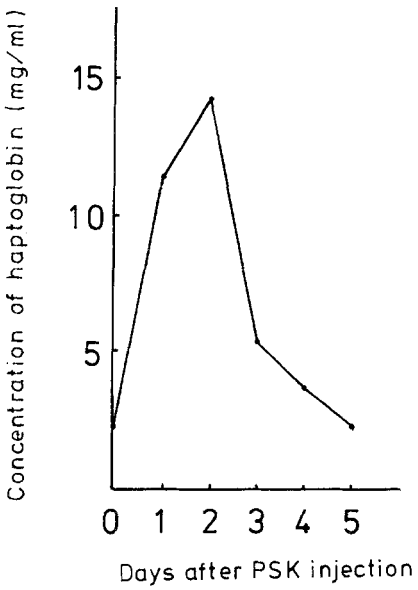

Figure 1. Time-dependent increase in the concentration of haptoglobin.

α(1-4)-linked glucan) [18, 19] or pustulan (a β(1-6)-linked glucan) [20, 21] did not increase the serum concentration of haptoglobin (data not shown).

In Vivo Inhibition of Tumor Growth by Haptoglobin

The effect of haptoglobin on the growth of Sarcoma-180 tumor cells *in vivo* was studied with the purified mouse haptoglobin. Each group consisted of six mice. As shown in Fig. 2, haptoglobin inhibited tumor growth by 44% when it was injected at a dose of 0.7 mg/mouse for five days. This inhibitory activity of haptoglobin against tumor growth was reproducibly observed in several independent experiments. However, growth inhibition of about 73% was seen when PSK was administered twice intraperitoneally at 12.5 mg/mouse on days 1 and 3. On the other hand, i.v. administration of 0.7 mg/mouse/day of bovine serum albumin (BSA) for five days did not inhibit the tumor growth.

Effect of Haptoglobin on Cytolytic Activities of Macrophages

As shown in Fig. 3, addition of haptoglobin resulted in a dose-dependent increase in the cytolytic activities of OK-Mϕ against C3MC2 tumor cells. However, TGC-Mϕ did not show cytolytic activity even in the presence of haptoglobin (data not shown), and haptoglobin itself was confirmed to have no effect on the tumor cells. The following studies were then performed to study the mechanism of the cytolytic activity of macrophages against tumor cells, which is increased by the addition of haptoglobin.

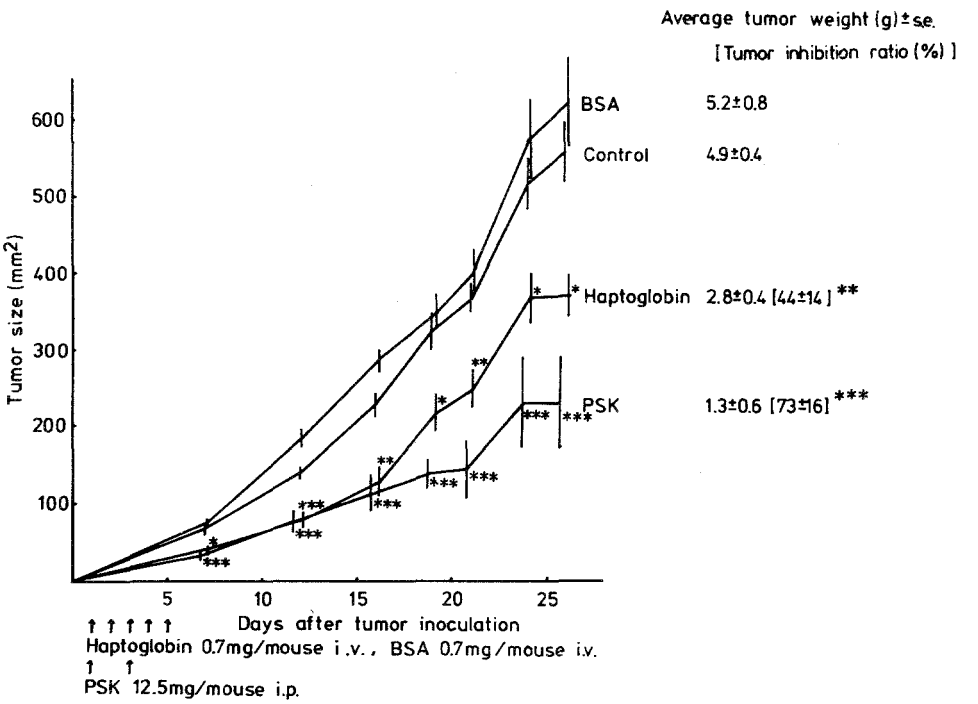

Figure 2. Effect of haptoglobin on Sarcoma-180-bearing ICR mice. Female ICR mice (6 mice/group), 7-9 weeks old, were inoculated subcutaneously with Sarcoma-180 cells (2×10^6 cells/mouse). Administration of samples was started 24 h after the inoculation of Sarcome-180 cells. Tumor weights were determined on day 26 after inoculation. Tumor weights are means ± standard error. * and ** are $P < 0.01$ and $P < 0.001$, respectively, when compared with the control by Student's t-test.

Effect of Haptoglobin on Activation of Macrophages

As shown in Fig. 4, the glucose consumption of PP-Mϕ increased dose-dependently on the addition of haptoglobin. This increased glucose consumption was approximately 1.5 times that of the control at maximum and the difference was statistically significant ($P < 0.05$). The effect of haptoglobin was much greater than those of hemopexin, ceruloplasmin [22] and transferrin [23] but less than that of LPS. The effect of haptoglobin on the production of superoxide anions was then examined with PP-Mϕ. As shown in Fig. 5, the ratio of cells that produced superoxide anions was remarkably increased by the addition of haptoglobin. But mouse hemopexin did not have this effect. Thus, mouse haptoglobin affected the activation of macrophages differently from mouse hemopexin. As another effect of haptoglobin on macrophages, the effect of haptoglobin on macrophage production of interleukin-1 was examined by measuring the IL-1 activity of the supernatant of PP-Mϕ cultured with or without LPS in the presence of absence of haptoglobin. As shown in Fig. 6, production of IL-1 by macrophages was maximally enhanced by the addition of 40 μg/ml haptoglobin either with or without LPS, but haptoglobin, itself was confirmed to have no activity similar to that of IL-1. Hemopexin did not have the same effect.

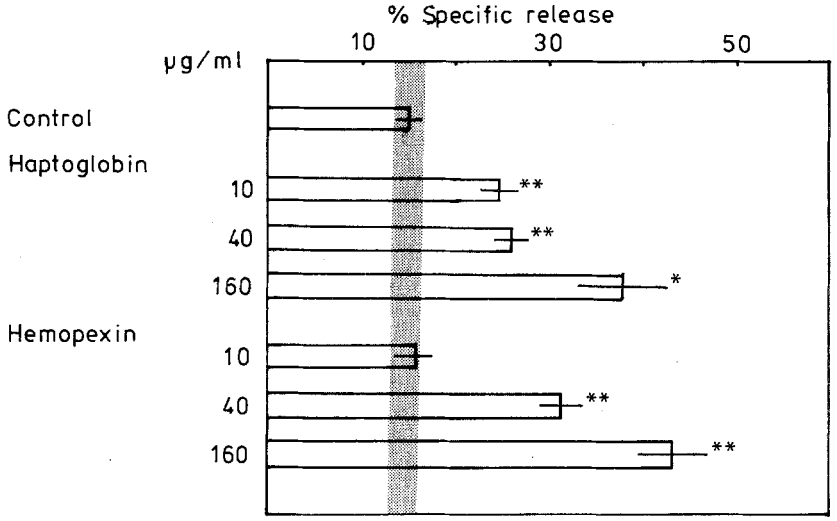

Figure 3. Effect of haptoglobin and hemopexin on the cytolytic activity of OK-432 elicited macrophages. The experimental details are given in the text. Each value is the mean of quadruplicate determinations with the standard error of the mean. * and **, significantly different from control experiments without the addition of the glycoproteins, shown by the shaded band, when compared by Student's t-test ($P < 0.02$ and $P < 0.01$, respectively).

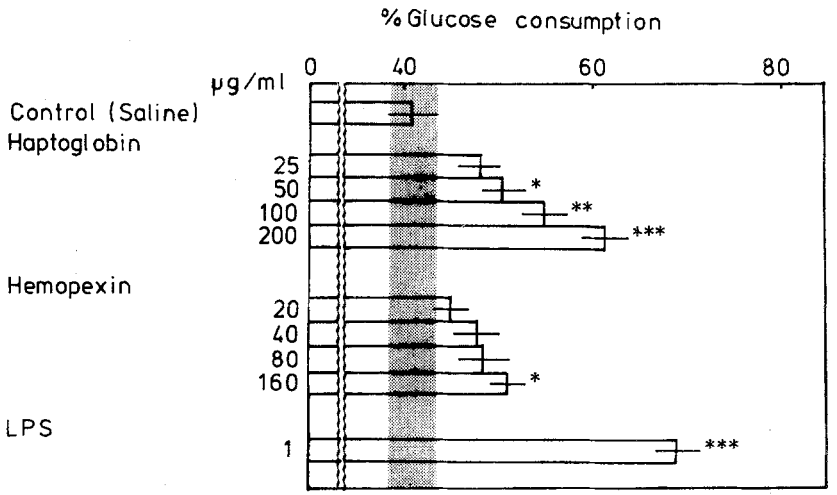

Figure 4. Effect of haptoglobin, hemopexin and LPS on glucose consumption of proteose-peptone-elicited macrophages. The experimental details are given in the text. Each value is the mean of quadruplicate determinations with the standard error of the mean. *, ** and ***, significantly different from control experiments without the addition of the glycoproteins, shown by the shaded band, when compared by Student's t-test ($P < 0.05$, $P < 0.02$ and $P < 0.01$, respectively).

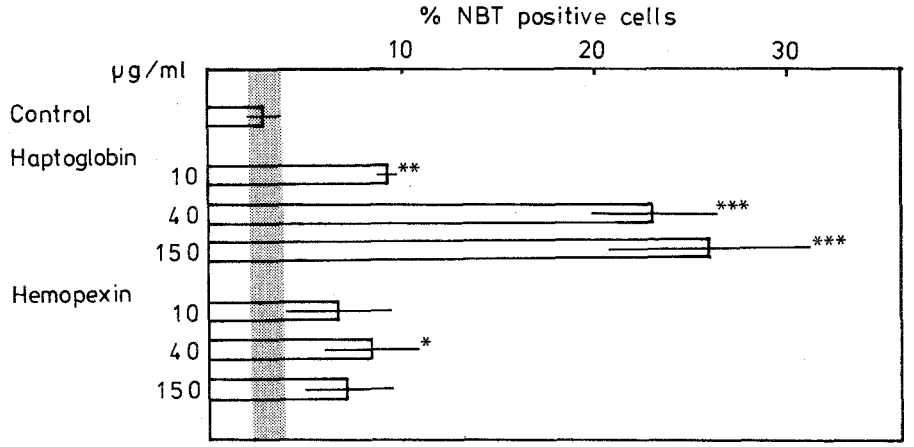

Figure 5. Effects of haptoglobin and hemopexin on production of O_2^-. The experimental details are given in the text. Each value is the mean of triplicate determinations with the standard error of the mean. *, ** and ***, signficantly different from control experiments without the addition of the glycoproteins, shown by the shaded band, when compared by Student's t-test ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively).

Effect of Haptoglobin on Cytotoxic T-Lymphocytes

Finally, the effect of haptoglobin on the cytolytic activity of cytotoxic T-lymphocytes against X-5563 tumor cells was examined. As shown in Fig. 7, induction of cytotoxic T-lymphocytes were greatly enhanced by the addition of haptoglobin at any effector:target ratio. These results suggest that haptoglobin is involved, not only in the activation of macrophages, but also in that of cytotoxic T-lymphocytes. Hemopexin also enhanced the induction of cytotoxic T-lymphocytes even though its effect was somewhat weaker than that of haptoglobin. However, haptoglobin and hemopexin themselves did not show a cytotoxic effect on the tumor cells at the concentrations used in these assays.

Discussion

In the present study we found that when PSK was injected intraperitoneally at 12.5 mg/mouse, the total concentration of haptoglobin in the serum increased to about seven times that found in the serum of control mice. Also in the case of human serum, it was reported that the concentration of haptoglobin increased in certain diseases to more than five times the concentration in normal serum [24]. Mouse haptoglobin inhibited tumor growth *in vivo* (Fig. 2) at an inhibition ratio of about 44%. A plausible explanation to why this value is lower than that in the case of the administration of PSK itself is as follows. We observed a significant dose response effect on the administration of haptoglobin at 0.7 mg/mouse for five days. However, PSK administration can induce much greater amounts of haptoglobin in serum as shown in Fig. 1. Furthermore, PSK administration may cause increases in a number of serum factors, one of which is haptoglobin, and these factors may enhance the activities of various immunocompetent cells.

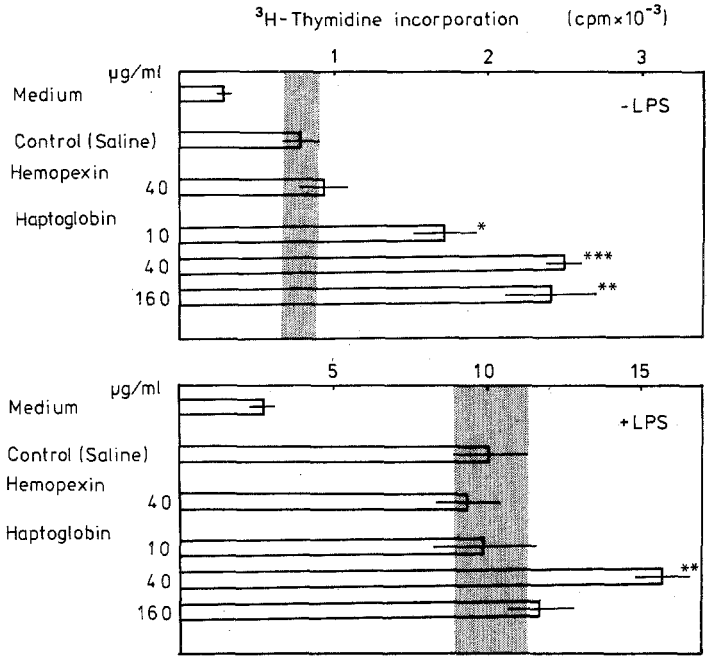

Figure 6. Effects of haptoglobin and hemopexin on production of IL-1 in the presence or absence of LPS. The experimental details are given in the text. Each value is the mean of quintuplicate determinations with the standard error of the mean. LPS was added at the concentration of 20 μg/ml (+LPS). *, ** and ***, significantly different from control experiments without the addition of the glycoproteins, shown by the shaded band, when compared by Student's t-test ($P < 0.02$, $P < 0.01$ and $P < 0.005$, respectively).

The anti-tumor effect of PSK was originally reported by Ohno *et al.* [25]. Ehrke [26] also reported that PSK had a potentiating effect on the immune response to allogenic antigens *in vitro*. In order to study the mechanism by which haptoglobin, one of the components that increases in the serum of mice to which PSK has been administered, inhibits tumor growth, we examined its effects on cells of the immune system, especially macrophages and T-lymphocytes. Mouse haptoglobin was found to enhance the cytolytic activities of macrophages against tumor cells *in vitro* (Fig. 3). We reported in a previous paper [8] that hemopexin increased the cytolytic activities of macrophages by enhancing the binding between macrophages and tumor cells. However, the mechanism by which haptoglobin increases the cytolytic activity of macrophages has not yet been elucidated. We want to investigate the different effects of haptoglobin and hemopexin on the activation of macrophages. There may be two possible mechanisms of macrophage activation. One is direct activation of cytolytic macrophages, and the other is indirect activation through suppression of suppressor macrophages. Lynch and Salomon [27] have reported that suppressor macrophages produce prostaglandins E_1 and E_2, which inhibit the appearance of cytolytic macrophages. Therefore, the effect of haptoglobin on macrophage production of prostaglandin E_2 was examined, but no inhibitory effect on the production was observed (data not shown), indicating that haptoglobin probably does not take part in the activation of macrophages through the inhibition of suppressor macrophages.

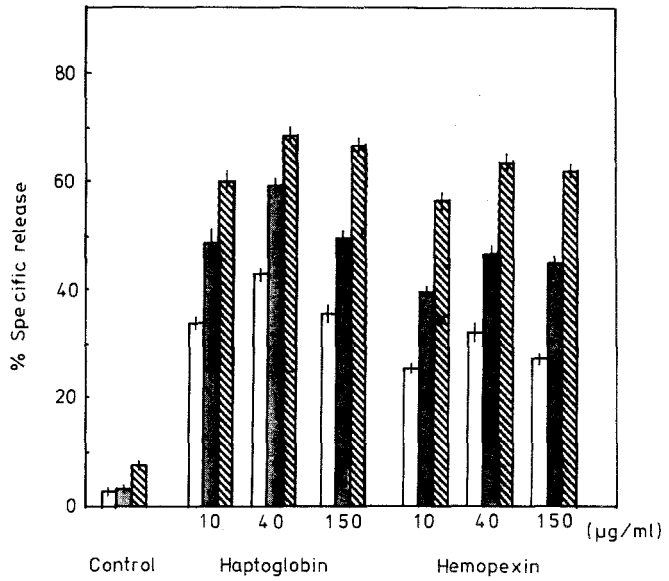

Figure 7. Effects of haptoglobin and hemopexin on the activity of CTL. The experimental details are given in the text. (●), Effector:Target (E/T) ratio = 28; (▲), E:T = 14;(■), E:T = 7. Each value is the mean of triplicate determinations with the standard error of the mean.

Regarding the direct activation of macrophages, addition of haptoglobin was shown to increase the glucose consumption of macrophages (Fig. 4) and to increase remarkably the ratio of cells that produce superoxide anions (Fig. 5). As the purified mouse haptoglobin employed in this study showed only a trace reaction (20 mg/ml) in the *Limulus* assay these effects cannot be due to contamination of the haptoglobin by LPS. Indeed haptoglobin itself probably directly activates macrophages in some way.

As for the increase in glucose consumption and the enhancement of superoxide anion formation, we obtained different results with hemopexin, suggesting that the mechanisms of action on macrophages could be different from those in the case of haptoglobin. Since Tsapis *et al.* [28] have reported that haptoglobin binds hemoglobin and increases its peroxidase activity, one possibility is that haptoglobin increases the superoxide anion formation of macrophages by binding myeloperoxidase in macrophages and enhancing the enzyme activity.

In order to investigate other aspects of the activation of macrophages by haptoglobin, its effect on the induction of IL-1 production by macrophages was examined. The addition of haptoglobin also enhanced IL-1 production (Fig. 6). Gery *et al.* [29] reported that IL-1 accelerates the proliferation of T-lymphocytes, so haptoglobin can be expected to have an effect on T-lymphocytes by enhancing IL-1 production. So, we examined the effect of haptoglobin on the induction of CTL and found that haptoglobin enhanced the generation of CTL (Fig. 7). This enhancement of cytotoxicity toward X-5563 cells may not be due to the activation of natural killer cells, because X-5563 cells are rather insensitive to natural killer cells (T. Okada, Y. Imai, T. Osawa; unpublished results).

These results together suggest that haptoglobin affects macrophages, enhancing their cytolytic activities by direct activation, and increases the induction of CTL possibly

through the induction of IL-1. This would result in an inhibitory effect on tumor growth *in vivo*. These effects of haptoglobin on macrophages are different from those of hemopexin, which is also increased in mouse serum after administration of anti-tumor polysaccharides and has been found to increase the attachment of activated macrophages to tumor cells [8]. It appears that these serum glycoproteins play somewhat complementary roles in maintaining homeostasis of the body and in natural resistance surveillance against tumors.

Acknowledgements

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