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Enhanced release of reactive oxygen intermediates by immunologically activated rat Kupffer cells

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SUMMARY

Release of O_2^- and H_2O_2 from isolated rat liver Kupffer cells was studied by making use of the methods of SOD sensitive ferricytochrome c reduction and horseradish peroxidase catalysed scopoletin oxidation, respectively. Kupffer cells from BCG treated rats showed a 1·8 times significantly higher O_2^- release and a 2·4 times higher H_2O_2 release as compared to the controls. Moreover the yield of Kupffer cells was also increased with administration of BCG. These results suggest that Kupffer cells can be immunologically activated to secrete larger amounts of O_2^- and H_2O_2.

Keywords Kupffer cells immunological activation superoxide anion hydrogen peroxide

INTRODUCTION

Kupffer cells (KC) are fixed tissue macrophages of the liver, and play a role in defence mechanisms, mainly in case of alimentary tract infection (Seljelid, 1980). The functional mechanisms of KC remain obscure. Recently, techniques for isolating KC have been developed (Munthe-Kass *et al.*, 1975; Toh, Yamamoto & Kikuchi, 1981) and KC functions such as phagocytosis, production of immunological mediators and antigen presentation have been reported (Toh *et al.*, 1981; Rogoff & Lipsky, 1979, 1980). In the oxygen-dependent anti-microbial system of mononuclear phagocytes the reactive oxygen intermediate (ROI), such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2), is a major weapon of phagocytes used for the killing of bacteria or for the cytolysis of tumour cells (Nathan *et al.*, 1979a, 1979b; Sasada & Johnston, 1980; Murray & Cohn, 1980). Therefore, it is important to investigate the oxidative metabolism of KC. Bhantnagar *et al.* (1981) reported for the first time, O_2^- release by zymosan stimulated rat KC. However, there have been no reports about activated KC related to the oxidative metabolism as yet. We now report O_2^- and H_2O_2 production by rat KC triggered with cytochalasin E and wheat germ agglutinin (WGA) or phorbol myristate acetate (PMA), and its immunological enhancement by Bacille Calmette-Guérin (BCG) administration.

MATERIALS AND METHODS

Animals. Male Wistar rats (Kyudo Co., Tosu, Saga, Japan), 8–12 weeks of age and weighing 200–250 g, were maintained under conventional laboratory conditions. Ten milligrams (4×10^8) of heat killed BCG (treated at 121°C for 15 min, Lot No. 1406M, Japan BCG laboratory, Tokyo,

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Japan) was given i.p., a booster dose of the same amount was given 3 weeks later and the rats were killed 7–10 days afterwards. Control rats were given 1 ml of 0·9% saline.

Preparation of autologous rat serum. Blood samples were obtained from ether anaesthetized rats by cardiac puncture, without an anticoagulant. The samples were kept at room temperature for 1 h, then at 4°C for 75 min and centrifuged at 800*g* for 15 min at 4°C. Serum was sterilized by passing through 0·45 μm Millipore filter (Millpore Co., Bedford, Maryland, USA), prepared into aliquots and stocked at −70°C until use.

Preparation of rat liver non-parenchymal cells (NPC). NPC was prepared according to Toh *et al.* (1981). All procedures were performed under sterile conditions. In brief, rat liver was perfused *in situ* with Eagle's minimal essential medium (containing 100 μg/ml Kanamycin and 250 μg/ml Cefazolin, Nissui Pharmacol. Co., Tokyo), then the liver was mechanically digested with 10 ml of Dulbecco's phosphate-buffered saline (D-PBS, pH 7·4) containing 0·1% collagenase (type I, Sigma Co, St Louis, Missouri, USA). After filtration and two washings, NPC was obtained with ultracentrifuge by Percoll density gradient (Percoll [Pharmacia Fine Chem. Co., Uppsala, Sweden]: twice concentrated PBS = 1:1) at 45,000*g*, for 60 min at 4°C. NPC was washed twice, and suspended in RPMI 1640 medium (GIBCO, Grand Island, New York, USA) containing 20% autologous rat serum, 200 u/ml penicillin and 200 μg/ml streptomycin and adjusted to $1{\cdot}0 \times 10^7$ cells/ml.

Preparation of adherent cells (KC). A NPC suspension (0·2 ml, 2×10^6 cells) was plated on a sterile 13 mm plastic cover slip (Lux Co., Newbury Park, California, USA), and incubation was carried out in humidified 5% CO_2 air at 37°C for 120 min. The non-adherent cells were removed by washing the cover slip three times with 0·9% saline at room temperature. For further collection of adherent cells (KC), 5 ml NPC suspension was poured into 60 mm plastic dishes (Falcon Co., Oxnard, California, USA), and the preparations were incubated for 120 min, in the same manner described above. The harvest of the adherent KC was performed by pipetting in ice cold PBS containing 0·02% disodium ethylene diaminetetra-acetate dihydrate (EDTA).

Identification of KC. Liver NPC mainly consisted of two types of mononuclear cells; large and small cells. To distinguish which kind of cell was KC, a phagocytosis test was performed as follows.

Phagocytosis *in vivo* NPC was prepared 18 h after i.v. administration of 0·2 ml of carbon colloid solution (carbon 2·5 mg/ml saline, Nakarai Chem. Co., Kyoto, Japan). In the specimen of Giemsa stained NPC, large and small mononuclear cells were present. The large but not small cells mainly phagocytosed the carbon particles (Fig. 1a).

Phagocytosis *in vitro* Adherent cells of NPC on cover slips were incubated with 0·2 ml of RPMI 1640 containing 20% autologous rat serum and 2×10^7 particles of latex (1·1 μm in diameter, Dow Chemical Co., Midland, Michigan, USA) for 1 h in a 5% CO_2 air humidified incubator at 37°C. After incubation the cover slip was washed throughly, dried and stained by the Wright Giemsa method. More than 90% of adherent cells were large mononuclear cells and most phagocytosed more than 10 particles of latex per cell. This also confirmed that the large adherent cells were KC (Fig. 1b).

Assay for ROI. **(i) O_2^- release by suspended cells** O_2^- was measured by the superoxide dismutase (SOD) inhibitable ferricytochrome c reduction method using cytochalasin E and WGA or PMA as triggers (Johnston, Godzik & Cohn, 1978; Nakagawara *et al.*, 1979; Nakagawara, Nathan & Cohn, 1981).

In the cytochalasin E and WGA triggering method, cells were suspended in 0·5 ml of HEPES (Dojin Chem. Co., Kumamoto, Japan)—saline (0·9% saline buffered with 5mM HEPES, pH 7·4, containing 2mM glucose, 1 mM $CaCl_2$ and 80 μM ferricytochrome c [type VI, Sigma]) at a cell concentration of $1 - 10 \times 10^6$ cells/ml. After pre-incubation at 37°C for 8 min, 5 μg/ml cytochalasin E (final concentration of dimethylsulphoxide [DMSO] was 0·25%, Aldrich Co., Milwaukee, Wisconsin, USA) was added, and 100 μg/ml WGA (Pharmacia Fine Chemical Co.) was also added 10 min after the addition of cytochalasin E.

In the PMA triggering method, cells were suspended in 0·5 ml of Krebs–Ringer phosphate solution with glucose (KRPG, 137 mM NaCl, 4·9 mM KCl, 1·2 mM $MgSO_4$, 0·5 mM $CaCl_2$, 5·7 mM Na_2PO_4, 5·5 mM glucose, pH 7·35) at the same cell concentration as in the cytochalasin E–WGA method and pre-incubated at 37°C for 7 min, then 100 ng/ml PMA (final concentration of DMSO was 0·033%, Sigma) was added. SOD sensitive cytochrome c reduction was detected using a two

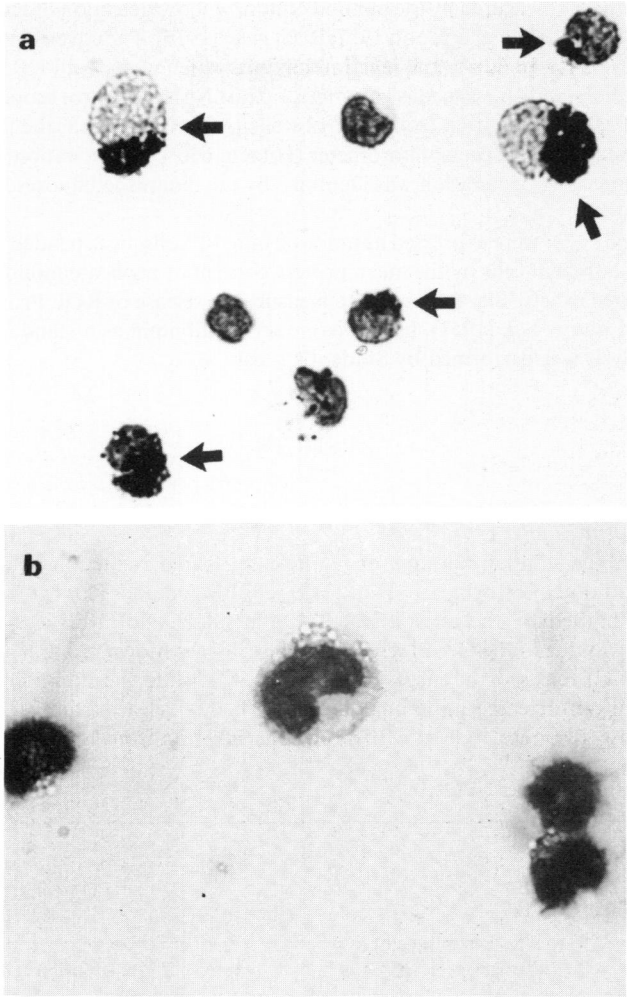

Fig. 1. Phagocytosis *in vivo* and *in vitro*. (a) Non-parenchymal cells were prepared 18 h after intravenous injection to male Wistar rats of 1 ml of colloid carbon solution (carbon 2·5 mg/ml). Large mononuclear cells (Kupffer cell) phagocytosed the carbon particles (indicated by arrow). Giemsa stain, magnification × 430. (b) Large mononuclear cells (Kupffer cell) adhering to a plastic cover slip were incubated with 0·2 ml of RPMI 1640 containing 20% autologous serum and 2×10^7 latex particles (1·1 μm in diameter). After 2 h incubation under humidified conditions with 5% CO_2 air, at 37°C, the cover slip was washed, dried and stained by the Wright–Giemsa method. Numerous latex particles were ingested in the cytoplasm of adherent Kupffer cells, magnification × 690.

wavelength spectrophotometer (Hitachi 557, Tokyo) at 550–540 nm. The molar extinction coefficient of cytochrome c was used as $19{\cdot}1 \times 10^3$/M.

(ii) O_2^- and H_2O_2 release by adherent cells O_2^-: the cover slip with adhering KC was incubated at 37°C for 60 min in a 24 well multidish (Falcon Co.) containing the reaction mixture. The solution was 0·75 ml of KRPG containing 80 μM ferricytochrome c, 100 ng/ml PMA and with or without 20 μg/ml SOD (Sigma). After incubation, the reduction of ferricytochrome c in the supernatant was measured with a spectrophotometer at 550 nm. O_2^- release was expressed as SOD sensitive cytochrome c reduction. The molar extinction coefficient of cytochrome c at 550 nm was used as $21{\cdot}0 \times 10^3$/M (Nakagawara *et al.*, 1981).

H_2O_2 production was measured by the method of fluorometric detection of scopoletin oxidation (Nakagawara *et al.*, 1981; Nathan & Root, 1977; Root *et al.*, 1975). The cover slip with adhering KC was incubated at 37°C for 60 min in the reaction mixture which was 1·5 ml KRPG containing 0·5 purpurogallin unit horseradish peroxidase (Sigma), 1·0 mM NaN_3, 5–20 nM scopoletin (Sigma) and 100 ng/ml PMA. PMA was omitted from control wells. After incubation, the fluorescence of the supernatant was measured by spectrofluorometer (Hitachi 650 10S), excitation beam was 350 nm and emission 460 nm. H_2O_2 generation was identified by the diminished fluorescence of scopoletin (Root *et al.*, 1975).

Data for O_2^- and H_2O_2 were expressed as nmol/60 min/10^6 cells in suspended cells, and nmol/60 min/mg protein in adherent cells by the mean protein content of triplicate monolayers of adherent cells that were treated exactly like those used to measure the release of ROI. Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Statistical analysis was performed by Student's *t*-test.

RESULTS

O_2^- release by resident Kupffer cells

Liver NPC showed substantial amounts of O_2^- release. Rapid reduction of cytochrome c was recognized after addition of cytochalasin E and WGA or PMA alone. This cytochrome c reduction was completely inhibited by the addition of 20 μg/ml SOD, indicating that the reduction of ferricytochrome c was due to the O_2^- released from the cells. As shown in Table 1, the adherent cell fraction (mainly KC) released the largest amount of O_2^-, while the non-adherent cell fraction (mainly endothelial cell) released a minimal amount. The O_2^- release from EDTA harvested KC showed a linear dose–response, at least with a cell concentration from 1–10 × 10^6 cells/ml (Fig. 2).

Table 1. O_2^- release from rat liver NPC

	Percentage of large cells (KC)	O_2^- release (nmol/min/5 × 10^6 cells)	Correlation value of O_2^- release by large cells (KC) (nmol/min/5 × 10^6 cells)
NPC	44%*	0·438 ± 0·033† (*n* = 5)	0·995 ± 0·076†
Adherent cells	66%	0·792 ± 0·119 (*n* = 3)	1·204 ± 0·182
Non-adherent cells	17%	0·215 ± 0·020 (*n* = 6)	1·264 ± 0·120

Cytochalasin E (5 μg/ml) and wheat germ agglutinin (100 μg/ml) were used as triggers.

* Mean of 10 high power fields, † mean ± s.e., *n* = number of experiments.

O_2^- releasing activity of BCG activated rat liver non-parenchymal cells

The yield of NPC from BCG treated rat was 3·78 ± 1·50 cells/liver, 2·8 times that of control (Table 2), while the percentages of large cells were similar. The O_2^- release by NPC obtained from BCG treated rats were about three times that of control rats.

O_2^- and H_2O_2 release from activated Kupffer cells

To determine whether the KC was activated, the release of O_2^- and H_2O_2 from adherent KC and EDTA harvested KC was measured (Table 3).

In adherent cells, O_2^- release from control rat KC was 189 ± 78 nmol/60 min/mg protein (mean ± s.d., *n* = 4, each value being the mean of triplicate or duplicate determinations), while the BCG treated rat KC level was 342 ± 68 (*n* = 4).

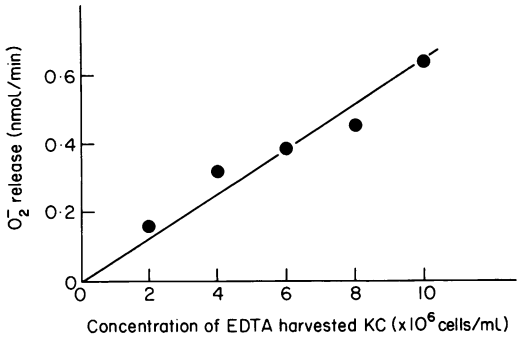

Fig. 2. O_2^- release by EDTA harvested Kupffer cells. The *r* value is 0·98. Sequential addition of cytochalasin E 5 μg/ml and WGA 100 μg/ml was used as a trigger. Cells were prepared by detaching the adherent ones which contained 66% large cells (Kupffer cell) and 34% small endothelial cells. The reaction condition was as described in the methods.

Table 2. O_2^- release by BCG activated rat liver NPC

	Cell yields of NPC $\times 10^7$ cells/liver	Percentage of large cells*	O_2^- release by NPC (nmol/min/10^6 cells)
Control (4)	1·35±0·41	30·5%	0·163±0·050
BCG (5)	3·78±1·50	31·4%	0·482±0·121†

PMA 100 ng/ml was used as a trigger.

(*n*) Number of experiments. Data are the mean±s.d. of each experiment using triplicate samples.

* Mean of indicated number of experiments, analysed using Giemsa stained specimens.

† Significant compared to control ($P<0·01$).

Table 3. O_2^- and H_2O_2 release by immunologically activated rat KC

	Adherent KC		EDTA harvested KC	
	O_2^- (nmol/60 min/mg protein)	H_2O_2	O_2^- (nmol/60 min/10^6 cells)	H_2O_2
Control (4)	189±78	123±11	6·04±1·70	4·08±0·60
BCG (4)	342±68*	294±83*	13·47±3·67†	11·35±2·63†

Data are the mean±s.d. Triplicate or duplicate samples were used in each experiment.

(*n*) Number of experiments.

Significant compared to control (*$P<0·05$, †$P<0·02$).

On the other hand, the H_2O_2 release from BCG treated KC was 294±83 nmol/60 min/mg protein ($n=4$), while that of the control was 123±11 ($n=4$).

In EDTA harvested KC, O_2^- release from control rat KC was 6·04±1·70 nmol/60 min/10^6 cells ($n=4$), while BCG treated rat KC showed 13·47±3·67 ($n=4$). H_2O_2 release by control rat KC was 4·08±0·60 nmol/60 min/10^6 cells ($n=4$), and those of BCG treated KC was 11·35±2·63 ($n=4$).

Thus O_2^- and H_2O_2 release from BCG treated rat KC was significantly higher than those of

control. KC from the BCG treated rat showed a significantly higher O_2^- release, 1·8 times higher in adherent cells and 2·4 times higher in EDTA cells as compared to those of control rat KC. BCG treated rat KC secreted a significantly higher H_2O_2, 2·4 times higher in adherent cells and 2·8 times higher in EDTA harvested KC than those of control rat KC.

The cell diameter of BCG treated rat KC was not significantly different from that of control KC. The mean protein content of untreated rat was $36·9 \pm 5·4$ $\mu g/10^6$ cells (mean $\pm$ s.d. of triplicate, $n=4$), and those of BCG treated rat KC was $37·7 \pm 4·8$ $\mu g/10^6$ cells ($n=4$). There was no significant difference between these two groups.

O_2^- release by resident KC on one cover slip was 1·5–2 nmol/60 min. Mean protein content of the resident KC on one coverslip was 7–12 μg. Thus the value of O_2^- release of EDTA harvested KC is similar to that of adherent KC.

DISCUSSION

The KC, fixed to the sinusoidal wall of the liver, are the largest family of so called 'tissue macrophages'. These cells have a great capacity to ingest exogenous particles, including bacteria, and therefore play a great role in host defence mechanisms (Seljelid, 1980). Munthe-Kass (1976) reported that the KC have a Fc and C3 receptor-mediated phagocytosis. Rogoff & Lipsky (1979, 1980) showed that the isolated guinea-pig KC possess the capacity to take up and present antigen to primed T lymphocytes for the induction of the secondary antigen specific proliferative response, and to support the mitogen-induced T cell proliferating response. Toh *et al.* (1981) reported that the isolated rat KC had cytotoxic effects, which were enhanced by i.p. injection of concanavalin A or lens curinaris haemagglutinin before KC preparation.

Bhantnagar *et al.* (1981) were the first to have observed the presence of O_2^- releasing activity in the rat KC during phagocytosis of zymosan particles, and suggested that O_2^- might be derived from NAD(P)H oxidase through the hexose monophosphate shunt similar to that of polymorphonuclear leucocytes or of peritoneal macrophages. We found that the i.p. administration of BCG enhanced the release of both O_2^- and H_2O_2 from rat KC, thereby indicating that KC could be immunologically activated with an increased capacity to release the ROI. This may be the first documentation of the activation of KC, as related oxidative metabolism. The term 'activated macrophage' fundamentally refers to enhanced anti-microbial activity. Increased ROI production means one phase of enhanced anti-microbial activity (Karnovsky & Lazdins, 1978; Cohn, 1978). Enhanced bactericidal and tumoricidal activities accompanied with increased O_2^- or H_2O_2 generation in mouse peritoneal macrophage have been reported (Sasada & Johnston, 1980; Nathan *et al.*, 1979a, 1979b). The importance of the oxygen-dependent mechanism of macrophage microbicidal activity was emphasized by the finding that the intracellular killing of *Toxoplasma gondii* by activated macrophages could be inhibited by ROI scavengers such a SOD or catalase (Murray *et al.*, 1979).

Crofton, Diesselhoff-den Dulk & van Furth (1978) and Diesselhoff-den Dulk, Crofton & van Furth (1979) showed that the origin of KC was blood monocytes in normal steady state or in acute inflammatory phase. Nakagawara *et al.* (1981) reported that ROI release by cultured human monocytes gradually declined during differentiation towards macrophage *in vitro*.

Increased O_2^- and H_2O_2 releases from mouse peritoneal macrophages or macrophages derived from cultured human monocytes have also been demonstrated by *in vitro* activation with lipopolysaccharides or lymphokines (Pabst & Johnston, 1980; Nakagawara *et al.*, 1982). KC is highly differentiated macrophage. Therefore, ROI release by resident KC might be low level, but could be enhanced by BCG administration.

Increased production of ROI by activated macrophages may be the result of synthesis of increased amounts of NAD(P)H oxidase or enhancement of its inherent capacity (Johnston *et al.*, 1978). Administration of BCG would lead to production of lymphokine like substances which may stimulate the oxidative enzymatic processes of the mononuclear phagocytes.

Morphological changes of KC with BCG treatment was not clear in our results. However, after BCG administration, the cell yield of KC increased about three times over findings in the control

rats. This observation supports the data of Diesselhoff-den Dulk *et al.* (1979) who noted that the KC yield was increased with administration of zymosan or *Corynebacterium parvum*. Therefore, the action of KC is promoted with increase in cell number and the individual cell function stimulated by the immunological activation.

Our study suggests that the activation of KC is probably accompanied by an enhanced oxidative metabolism, and if such is indeed the case, would explain a part of the molecular basis for the activated KC.

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