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Citation	日本外科宝函 (1986), 55(5): 643-652
Issue Date	1986-09-01
URL	http://hdl.handle.net/2433/208649
Right	
Type	Departmental Bulletin Paper
Textversion	publisher

原 著

Development of a Bioassay of Opsonic Activity for Kupffer Cell and Humoral Factors Stimulating Phagocytosis

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Received for Publication, June. 30, 1986.

Abstract

Using the primary culture of rat Kupffer cells which maintain the specific function of mononuclear phagocyte in vitro, a bioassay of opsonic activity was developed. As phagocytizable material, ^{51}Cr -endotoxin was employed because of its biological nature that endotoxin is exclusively phagocytized by Kupffer cells and possesses a variety of pathogenetic roles.

The procedure of the assay was as follows. Twenty-four hours after initial plating of isolated Kupffer cells (5×10^5 cells) into the dishes with Eagle's Minimum Essential Medium (MEM) containing 10% fetal calf serum (FCS), the culture medium was replaced with MEM containing ^{51}Cr -endotoxin and test (or control) plasma. One hour later, the radioactivity in the cultured cells was measured. The ratio of the radioactivities in the cells incubated with test plasma to those incubated with control plasma was expressed as the opsonic index.

This index was not affected by opsonic proteins such as IgG, complement components, or plasma fibronectin. This finding suggests that unknown humoral substances enhancing the opsonic index are present in the fraction of 50%–60% saturated ammonium sulfate precipitates.

Introduction

Kupffer cells, which comprise the many portion of the reticuloendothelial system (RES), play an important role in host defense function by clearing and inactivating blood-borne bacteria, endotoxin, and nonbacterial particles. Therefore, the suppression of Kupffer cell function weakens the host defense, frequently leading to serious septic complications and further multiple organ failure^{1, 7, 10, 11, 15, 18, 19}.

Key words: Kupffer cells, Reticuloendothelial host defense, Bioassay of opsonic activity, Opsonic index, ^{51}Cr -endotoxin.

索引語: クッパー細胞, 細胞内皮系による生体防御, オプソニンの生物学的測定法 オプソニン指数, ^{51}Cr -エンドトキシン.

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It is well known that phagocytosis mainly depends on the humoral opsonic activity^{8,9} which is generally evaluated by a bioassay technique. A bioassay of opsonic activity in the RES is performed with the method using rat liver slices and ¹²⁵I-gelatinized lipid emulsion devised by *Saba et al.*^{20,21,22,26}. They found that bioassayable opsonin measured with this method correlates to the plasma fibronectin level^{4,23}. However, plasma fibronectin exhibits opsonic activity by binding to denatured collagen-coated particles, cytoskeletal debris, fibrin-fibrinogen complexes, and other nonbacterial particles^{25,27}, but not bacterial and non-gelatinized particles. Thus, the opsonic activity determined with Saba's method may not necessarily regulate RES phagocytosis for many kinds of bacteria and endotoxin.

In the present study, we describe a bioassay using the primary culture of rat Kupffer cells and ⁵¹Cr-endotoxin as the phagocytosable material. Additionally, evidence is presented indicating that there exist unknown substances stimulating Kupffer cell phagocytosis in human plasma.

Materials and Methods

Cell preparation and culture

Male Wistar rats weighing 200–300 g were used. Kupffer cells were isolated by the enzymatic perfusion technique^{12,13} as follows. The liver was perfused via the portal vein with Ca²⁺ free Hank's solution containing 5 mM GEDTA for 5 min and subsequently, with a 0.05% (w/v) collagenase (Sigma, St Louis, Mo.) solution containing 20 mM HEPES for 15 min. Next, the liver was extirpated and minced. The dissociation of liver cell cords into isolated cells was obtained by stirring the cell aggregate in a flask. The cell suspensions were filtered through a wire mesh (28 μ m pore size) to eliminate cell aggregates. Nonparenchymal cells were obtained by incubating at 37°C in the presence of 0.1% pronase (Merck, West Germany). All parenchymal cells were destroyed within an hour under these conditions. Nonparenchymal cells (1×10^6 cells) were inoculated into plastic dishes (Falcon 3001, Falcon, Oxnard, Calif.) in MEM (Gibco Laboratories Grand Island, N.Y.) supplemented with 10% FCS (Gibco) and 100 u/ml penicillin (Gibco). After the incubation at 37°C under 5% CO₂ in air for one hour, the cells in the dishes were repeatedly washed with phosphate buffer saline, and consequently, adherent cells, consisting almost exclusively of Kupffer cells, were obtained.

Identification and characterization of cultured Kupffer cell

Morphological observation was carried out with phase contrast microscopy and Giemsa staining. The phagocytic function of the cultured cells was studied by the phagocytosis of colloidal carbon (c11/143/a, Gunther-Wagner, Hanover, West Germany) and *Salmonella enteritidis* (gift from the Department of Microbiology, Kyoto University School of Medicine) which is specifically phagocytized by mononuclear phagocytes. Moreover, inhibitory action of NaF and NaN₃ on the phagocytosis was examined to study whether the energy necessary for the phagocytosis is produced by glycolysis, Embden-Meyerhof pathway. Fifteen minutes after the addition of NaF or NaN₃ at concentrations of 0.1 mM, 1 mM and 10 mM, phagocytosis of cultured Kupffer cells was quantitated.

Quantitation of the phagocytosis

Endotoxin (*E. coli*, LPS, 026, B6 Difco Laboratories Detroit, Mich.), used as the phagocytizable material, was labeled with $\text{Na}_2^{51}\text{CrO}_4$ (^{51}Cr) according to Braude's method⁵. The ratio of ^{51}Cr to endotoxin was 1 mci to 100 mg. The phagocytosis of the cultured cells was measured as follows: ^{51}Cr -endotoxin at a dose of 100 μg was added to the culture containing various concentrations of fresh plasma. After 15, 30, 45, 50, 120, 180 and 240 min, the cultured cells were washed thoroughly with phosphate buffer saline, then the cells were desquamated with a rubber policeman and harvested, and their radioactivities were determined by an autogamma spectrometer (Nuclear Chicago Corp., Des Plaines, Ill).

Analysis of humoral factors stimulating Kupffer cell phagocytosis in human plasma

Pooled human plasma from five healthy adults was precipitated with 40, 50 or 60% saturated ammonium sulfate (SAS). After removal of ammonium sulfate by dialysis, the effect of each supernatant of SAS-precipitated plasma on the phagocytosis was determined. In addition, the concentration of IgG, complement components, plasma fibronectin and several acute phase reactants in the supernatant was measured by single radial immunodiffusion.

Results

Figs. 1 and 2 show the phase-contrast microscopic findings of Kupffer cells phagocytizing colloidal carbon, and isolated Kupffer cells, respectively.

Figs. 3 and 4 show Giemsa staining of the cultured Kupffer cells with a large mononucleus, and those phagocytizing *Salmonella enteritidis*, respectively.

The correlation between the phagocytosis and dose of opsonin added to the cultures is shown in Fig. 5. The phagocytosis of the cultured cells was positively correlated with the concentration of opsonin. However, the linear increase in the phagocytosis disappeared at fresh plasma concentrations greater than 33%.

Fig. 6 shows the correlation between the phagocytosis and incubation time of cultures. Until one hour after the incubation at 37°C, the radioactivity in the cells increased linearly, but thereafter, the rate of increase declined.

Tabl 1. Content of various opsonic proteins in whole plasma and supernatant of 50% SAS precipitation.

	whole plasma	supernatant of 50% SAS precipitation
IgG	(mg/dl) 1350 ± 130	ND
Complement C3	(mg/dl) 17 ± 12	<3
C-reactive protein	(mg/dl) ND	ND
haptoglobin	(mg/dl) 210 ± 40	<10
ceruloplasmin	(mg/dl) 19 ± 2	<3
fibrinogen	(mg/dl) 172 ± 15	<20
fibronectin	(μg /dl) 280 ± 53	ND

ND: not detected

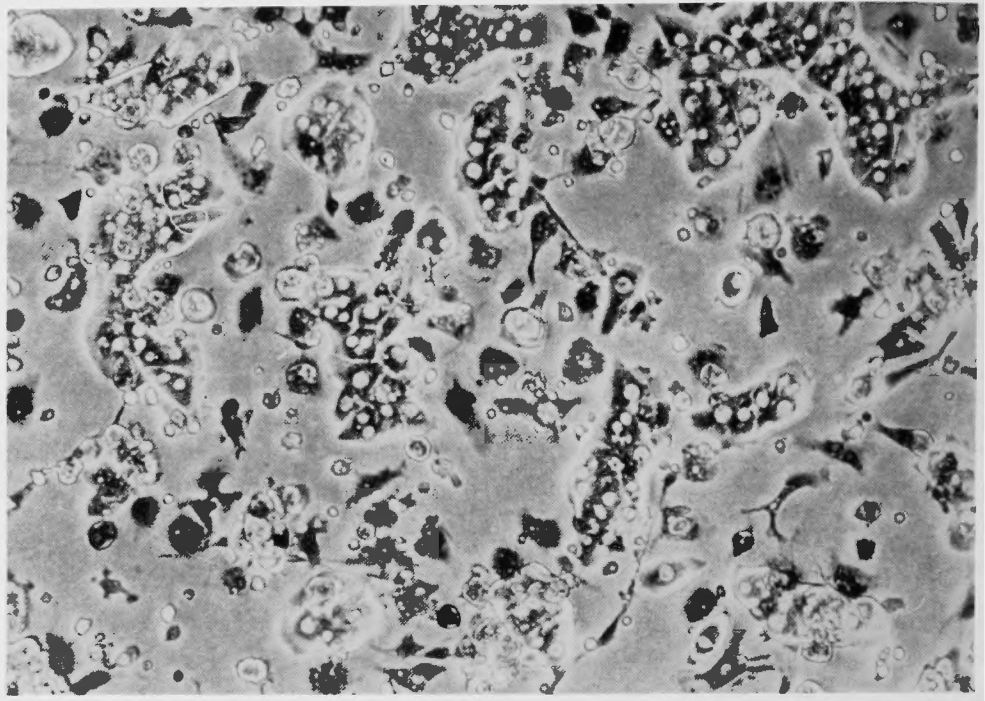


Fig. 1. Co-culture of hepatocytes and non-parenchymal cells obtained from rat liver. Hepatocytes are contacted with each other, and Kupffer cells phagocytize black carbon particles.

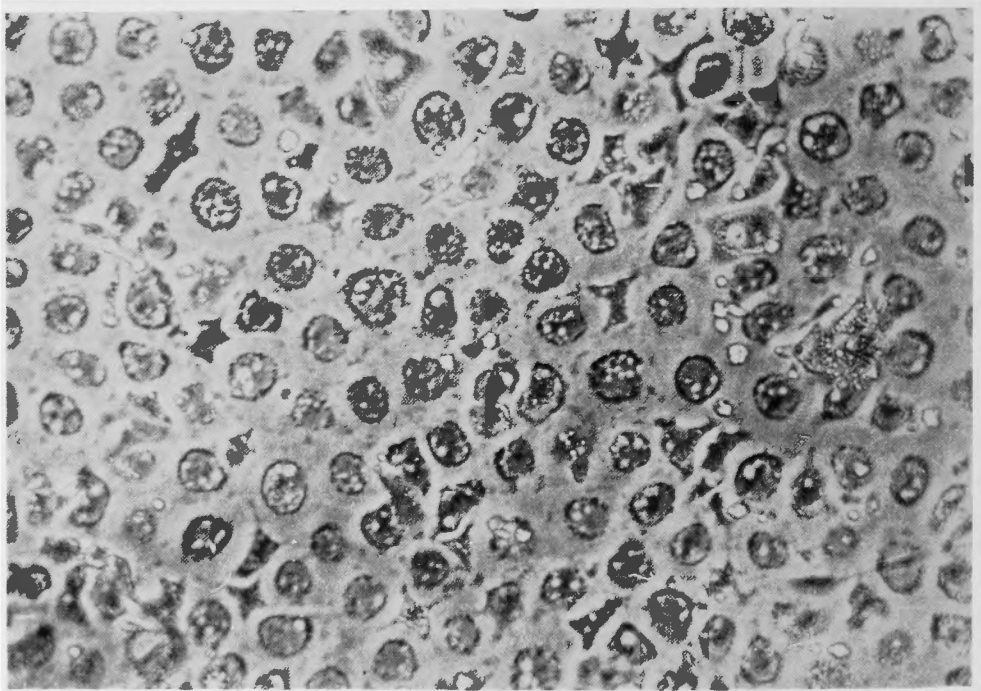


Fig. 2. Purely isolated Kupffer cells. Many granules and vacuoles are seen in the cytoplasm of Kupffer cells.

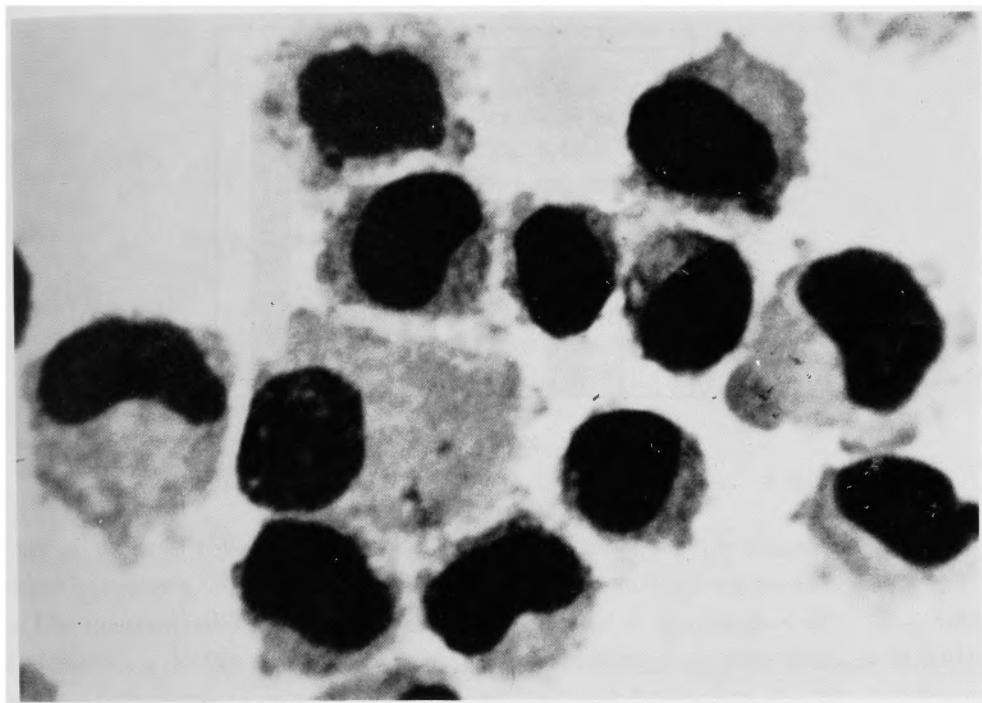


Fig. 3. Giemsa staining of Kupffer cells.

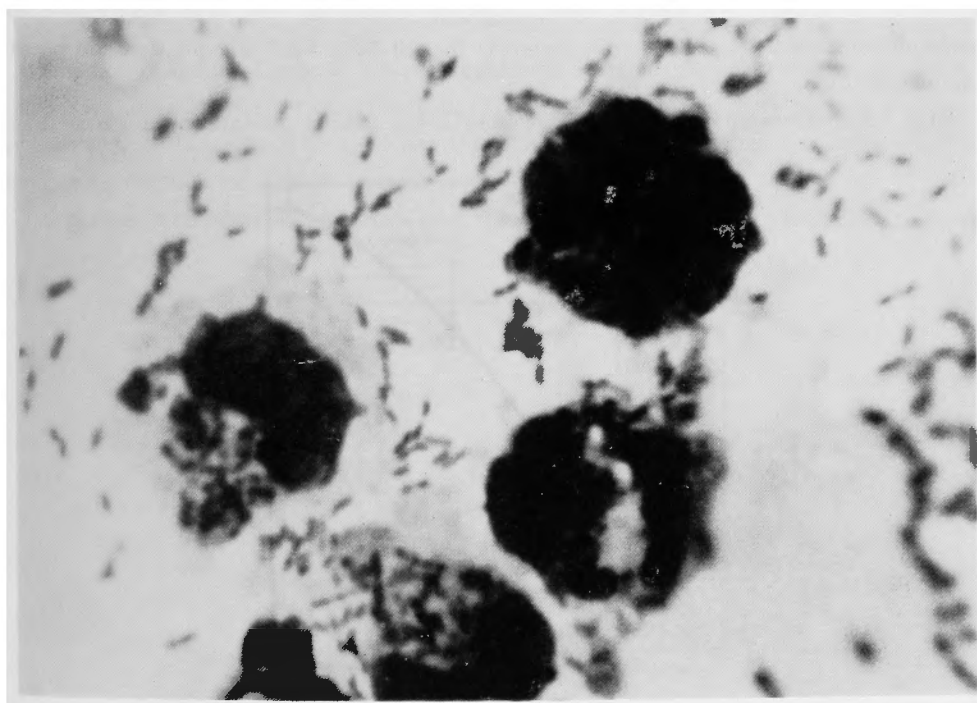


Fig. 4. Giemsa staining of Kupffer cell phagocytizing *Salmonella enteritidis*.

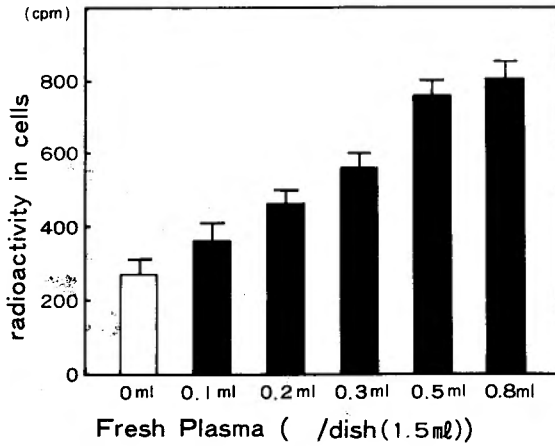


Fig. 5. Dose-dependent effect of opsonin on Kupffer cell phagocytosis.

Thus, the optimal conditions to measure opsonic activity was decided as shown in Fig. 7.

Fig. 8 demonstrates the inhibitory effect of NaF and NaN₃ on the phagocytosis of cultured Kupffer cells. The radioactivity in the cells preincubated with NaF concentrations of 1 mM and 10 mM was decreased to approximately 60% and 30% of the control, respectively; the radioactivity in the cells pre-treated with NaN₃ was decreased to a lesser extent.

Fig. 9 illustrates the stimulatory effect of human plasma treated with ammonium sulfate precipitation on Kupffer cell phagocytosis. The supernatant of 50% SAS precipitation dose-dependently enhanced the phagocytosis, whereas that of 60% SAS precipitation had no effect on the phagocytosis.

Table 1 demonstrates the concentration of IgG, C₃, plasma fibronectin, and several acute

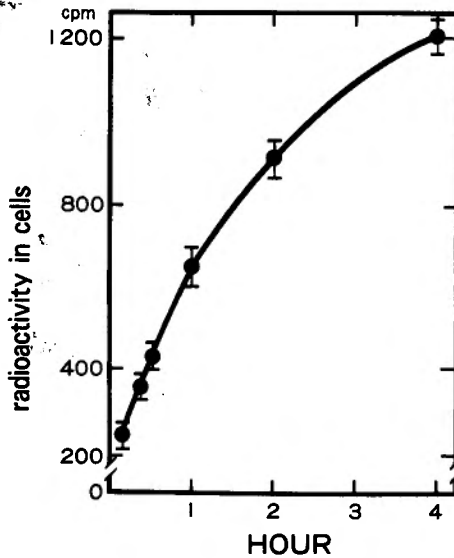
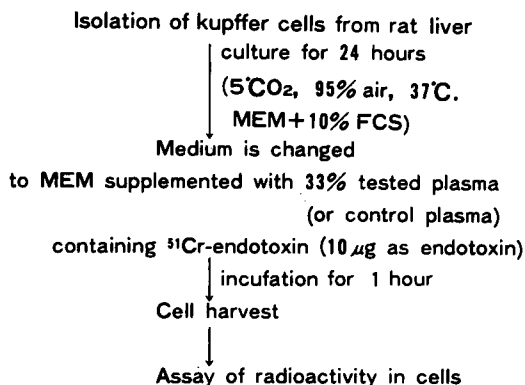


Fig. 6. Time course of ⁵¹Cr-endotoxin phagocytosis by Kupffer cells.

Assay of Opsonic Activity



Opsonic Index

$$\text{Opsonic Index} = \frac{\text{radioactivity in cells cultured with tested plasma}}{\text{radioactivity in cells cultured with control plasma}}$$

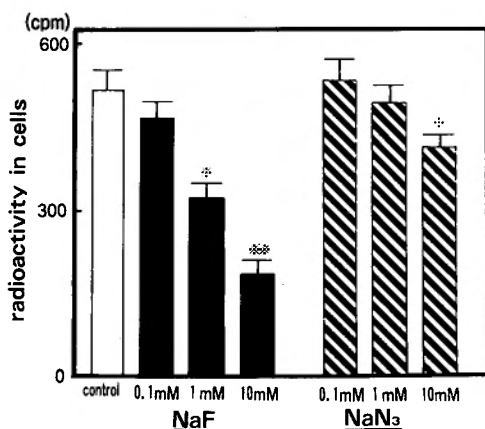
Fig. 7.

phase reactants in the supernatant of 50% SAS precipitated plasma and whole plasma, suggesting that none of these proteins is a factor stimulating Kupffer cell phagocytosis for ⁵¹Cr-endotoxin.

Discussion

Evaluation of humoral opsonic activity regulating Kupffer cell phagocytosis may be indispensable for estimating the reticuloendothelial host defense, because Kupffer cells account for more than 80% of the entire RES³⁾. From this point of view, the author developed the present method using the isolated Kupffer cells.

The primarily cultured Kupffer cells have the same biological activity with high phagocytic



* P<0.01, *† P<0.001 compared with control

Fig. 8. Inhibitory effect of NaF and NaN₃ on Kupffer cell phagocytosis.

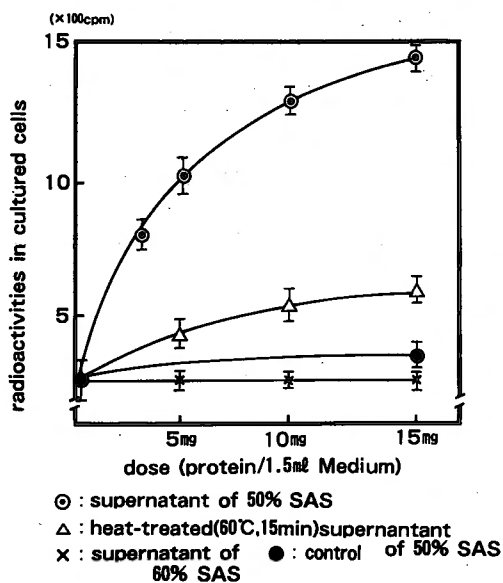


Fig. 9. Stimulatory effect of human plasma treated with ammonium sulfate precipitation on Kupffer cell phagocytosis.

capacity, while in Saba's method, it is not easy to obtain liver slices with exactly similar characteristics. The isolated Kupffer cells were identified by morphological observations and phagocytic activity for colloidal carbon, and *Salmonella enteritidis*. In addition, the cells had biochemical characteristics indicating a kind of macrophage in which phagocytosis was remarkably suppressed by NaF, an inhibitor of Embden-Meyerhof pathway and minimally affected by NaN_3 , an inhibitor of electron transport in the TCA cycle²⁴). Another characteristic of the present method is that ^{51}Cr -endotoxin was employed as a phagocytizable material instead of ^{125}I -gelatinized lipid emulsion, because endotoxin has a variety of pathogenetic roles in inducing renal failure, respiratory insufficiency and disseminated intravascular coagulation;^{6,14,28} moreover, endotoxin is specifically phagocytized by Kupffer cells, but not by other nonparenchymal cells in the liver¹⁷).

The opsonic activity measured with the present method does not correlate with the plasma fibronectin level, in contrast to Saba's method. This discrepancy between Saba's method and the present method is mainly due to the phagocytizable materials used, because fibronectin has an affinity for gelatin, thereby exhibiting its opsonic function to gelatinized lipid emulsion, but not endotoxin^{25,27}).

Using the present method, the opsonic indexes in 67% partially hepatectomized rats were measured as reported previously²). An increase in the opsonic index was observed after the partial hepatectomy, suggesting a compensatory response of the host defense system for maintaining RES phagocytosis at normal levels. Such enhancement in the opsonic activity could not be detected by Saba's method. *Saba et al.* noted that the opsonic activity was always decreased after trauma, burn, and surgery, followed by rapid restoration of normal levels unless

bacteremia and/or sepsis intervenes^{22, 23, 26}). Furthermore, in the experimental liver injury, it was found that the opsonic indexes varied with the degree of liver damage. In the rats with moderate liver injury, a high opsonic index was observed, being similar to that of partially hepatectomized rats²⁹). On the other hand, in the rats with severe liver injury such as galactosamine-induced fulminant hepatitis, the opsonic index was remarkably decreased. Thus, the opsonic index determined here represents the functional state of RES, and appears to be a prognostic indicator of liver injury.

Moreover, this assay system suggests the presence of unknown humoral substances, which enhance the opsonic activity, in the supernatant of 50% SAS precipitation of human plasma. In general, humoral factors stimulating the phagocytosis consist of opsonin and acute phase reactants^{8, 9, 16}). However, opsonic proteins such as IgG, complement components, plasma fibronectin, and various acute phase reactants were not detected in the supernatant of 50% SAS precipitation, or in only negligible quantities. Although, it is possible that the inhibitory factors were removed by SAS precipitation, a dose-dependent increase in the phagocytosis induced by the addition of the supernatant to the culture strongly suggest that the supernatant contains the phagocytosis-stimulating substances.

Acknowledgement

I would like to appreciate the great direction of Professor and Chairman, Dr. Takayoshi Tobe, and helpful suggestion of Professor and Chairman, Dr. Kazue Ozawa, Dr. Hidenari Takasan, and Dr. Masafumi Shibagaki. This study was presented at the 84th annual Congress of Japan Surgical Society, the 69th annual Congress of Japanese Society of Gastroenterology, and the 20th annual Congress of Japan Liver Society.

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和文抄録

クッパー細胞貪食能におけるオプソニン活性測定法の 開発に関する研究

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有 井 滋 樹

著者らは、従来の Saba らの肝組織片と ^{125}I -gelatinized lipid emulsion を用いた測定法に代わり、ラット初代培養クッパー細胞によるオプソニン活性測定法を開発した。

本法における培養クッパー細胞は旺盛な貪食能を有しており、肝組織片の生物学的活性や、その同一性に関する問題点を解決するものと考えられる。また、被

貪食異物としては病因論的意義を有し、かつ、生体においては殆んど特異的にクッパー細胞で摂取される ^{51}Cr -endotoxin を用いた。

さらに、本測定法により、既知のオプソニン因子とは異なる液性因子が50-60%飽和硫酸塩析分画に存在することが強く示唆された。