Phagocytic Activity and Hexose Monophosphate Shunt Activity of Cultured Human Kupffer Cells upon Zymosan, Erythrocytes, Amoebic Trophozoits, Latex Beads and Nitroimidazole

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Abstract

Abstract: Phagocytic function of human Kupffer cells was altered by the addition of nitroimidazole and amoebic trophozoits. The hypothesis was that phagocytic activity was dependent on the nature of additives in culture. The Kupffer cells were fractionated and lysosomes were isolated for enzyme estimations in presence of additives. Phagocytic activity function of human Kupffer cells was altered by the addition of nitroimidazole and amoebic trophozoits in Kupffer cell culture. The human Kupffer cells showed take up of zymosan particles larger than 1 micron in diameter and inert interaction with latex particles. The liver endothelial cells or phagocytes constitute a second line of defense in the liver for the phagoctosis function. The nitroimidazole slowed down the Kupffer cells phagocytosis activity of engulfing beads, trophozoits and removing the foreign materials from the vicinity while Kupffer cell phagocytic function remained totally intact. The potential role of Kupffer cells was phagocytosis and nitroimidazole slowed down the further activated Kupffer cells under physiologic conditions when Kupffer cells are active in clearing foreign substance from the circulation. In conclusion, theses first Kupffer cell and trophozoits interaction in the presence of nitroimidazole suggested the synergistic interaction and this cellular interaction in liver.

Key words: nitroimidazole, Kupffer cells, liver, phagocytosis

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[2] The Kupffer cells were harvested from the human liver biopsy samples recruited for ongoing research study on hepatic abscess and imidazoles. The present study was part of Ph.D dissertation "The effect of nitroimidazole on human liver cells during development of amoebic liver abscess" submitted to LLRM Medical College, Meerut

Introduction

Liver is made of parenchymal hepatocytes and nonparenchymal Kupffer cells. Nonparenchymal cells also contain other cells known as ito cells and fat storing cells[1]. The main function of nonparenchymal cells is to wipe out foreign bodies by suicidal action or phagocytosis. The nonparenchymal cells known as macrophages are fixed cells for defence as sinusoidal bags filled with mainly lysosomal enzymes in the vicinity of foreign stimulants or additives. The additives may be neutral particles, live cells or polymer beads to characterize the nature of phagocytes. The Kupffer cells were widely reported in experimental animals to evaluate the drug therapeutic monitoring using lysosomal enzymes [2], hepatocellular defense [3,4,5]. There is paucity of information of Kupffer cells phagocytosis data in human liver tissue perhaps due to two reasons. First, due to lesser yield of Kupffer cells from small liver biopsy sample and second reason due to less well defined phagocytosis behavior in presence of inert and avirulent additives. The lysosomal enzymes are widely known as macrophage function to evaluate the nature of phagocytosis. The parasites have been reported as mixed with nonparenchymal cells in cultures and stimulated immunosensitive release of cytokines, interleukins, VCAM, ICAM molecules at the site of interaction [6]. Phagocytosis in rat Kupffer cells is reported as consequence of activation of Kupffer cells [7-12]. The nitroimidazole is a antiparasitic chemical used in hepatic amoebiasis, giardia, trypanosome and other intestinal infections. Its nitro active group in imidazole compound provides the therapeutic action by stimulating phagocytosis and lysosomal enzyme release [13]. In present study, human Kupffer cells were exposed to zymosan, erythrocytes, amoebic trophozoits, latex beads and nitroimidazole additives to characterize the phagocytosis action of liver macrophages in isolated *in vitro* cultures. The study offers a quantitative evaluation of sinusoidal function of Kupffer cells to compare the interaction of different types of foreign bodies with live liver macrophages. The present study also answers the issue of what stimulation of nitroimidazole can be over liver macrophages. The novelty of this study is that it distinguishes the response of Kupffer cells for bioactive and inert foreign bodies as surface sensitive defense mechanism.

Materials and Methods

The procedures of liver biopsy excision and preparation of liver cells were followed as per ethical committee of institute.

Reagents:
The collagenase enzyme was purchased from Sigma-Aldrich, St Louis, MO and pronase Enzyme was purchased from Boerhinger Meinheim GMbH.

Isolation and preparation of Kupffer cells: The liver biopsy samples (6-10 grams) were digested with collagenase enzyme (Sigma-Aldrich, St Louis, MO) 15 mg/ml for 10 minutes and bottom pallet was used for hepatocyte experiments. Other remained supernatant part of digested liver was further digested with 2 mg/ml pronase enzyme (Boerhinger Meinheim GMbH) in 0.2 % Kreb's Hansleit Buffer pH 7.4 containing 5 mm glucose and 3 mM calcium chloride after separation of hepatocytes. The said suspension was incubated for 45 minutes at 37˚C stirring 250 revolutions per minute. After incubation, the cell suspension was filtered through nylon sieve 79 x 79 microns and filtrate was centrifuged and washed twice with KHB containing 5 mM glucose and 3 mM calcium chloride pH 7.4. After centrifugation and final washing the filtrate was centrifuged at 350 x g in ice cold centrifuge. The settled pallet at bottom was washed with TC 199 medium. The number of Kupffer cells was counted on Naubourgh counting chamber and visibility was determined by trypan blue [14].

Kupffer cell fractionation: The isolated Kupffer cells were divided in two parts. One part was used for Kupffer cellular enzymes and other part was used for in vitro experiments. For enzyme estimations Kupffer cells from freshly harvested preparations were sonicated at zero degree temperature in said KHB buffer in soniprep. Later these were fractionated for different cellular fractions as described following by Alabraba et al.[14, 15].

1. In refrigerated centrifuge above soniprep preparations were centrifuged at 1000 x g to take out cell nuclear fraction and again centrifuged at 9000 x g to take out mitochondrial fraction as pallet at bottom.
2. Above supernatant was centrifuged at 23000 x g to isolate microsomal bodies fraction along with fraction rich in peroxisomes.
3. The light turbid supernatant was centrifuged at 100000 x g to isolate lysosomal fraction as clear white pallet at bottom. Sometimes microsomes were contaminated with lysosomes. The lysosomal purity evaluation was used to exclude microsomes free from lysosomes.

Kupffer cells in cultures: The Kupffer cell cultures were maintained for 48 hours after isolation from pronase perfused liver biopsy samples. The in vitro cultured cells were screened for trypan blue exclusion [16]. The Kupffer cells were used for their in vitro phagocytic action upon foreign particles which were sheep erythrocytes, latex beads and zymosan suspended in 10 mM phosphate buffer saline pH 7.4 at 4°C. After harvesting Kupffer cells were added in phosphate buffered saline pH 7.4 containing 4.6 x 10^6 adherent cells and three ml PBS-TC 199 medium in culture flasks containing Kupffer cells in mediukm with particle cell ratio 1:5 for erythrocytes, 1:40 for latex beads and 1:4 for trophozoits. The ratio for zymosan and cell was 20:1. They were kept up to 48 hours or more in carbogen atmosphere (95 % O_2 and 5% CO_2) in desiccator with twice changes of medium after every 24 hours [16].

Additives: Zymosan was purchased from Sigma Aldrich, St Louis, MI. Zymosan unopsonized solutions in PBS were prepared and stored at -20 C as described by Bonney et al [9]. For each experiment, zymosan was thawed and sonicated for 1 min in sonicator (Branson Astrason 6 model). The sheep red blood cells (sRBC) were prepared as described by [10](Wright Silverstein 1984). The Kupffer cells as monolayers on cover

slip were washed, overlaid with 3 x 10^5 erythrocytes, dipped in PBS buffer and incubated for 45 minutes at 37 C

Phagocytosis Assays: Phagocytic activation was measured by estimating βglucuronidase, lactate dehydrogenase and plasminogen activator in above mentioned Kupffer cells. βGlucuronidase and lactate dehydrogenase were estimated as described in following section.

β-Glucuronidase enzyme assay: βGlucuronidase enzyme was measured by taking 5 mM 4-methylumbelliferyl βGlucuronide and 0.8 mg/ml bovine serum albumin in 125 ul of 160 sodium acetate buffer pH 4.8 in cuvette and termination by adding 1.8 ml 320 mM glycine with 200 mM soium carbonate buffer pH 10.5 after 5 minutes at 25˚C. The absorbance A405 was recorded. One unit enzyme liberated 1 μmole of 4-methylumbelliferone [17].

Lactate dehydrogenase assay: The lactate dehydrogenase enzyme was measured by taking 2.7 ml of 0.1M phosphate buffer pH 7.4 taken in 1 cm cuvette and added 0.1 ml Kupffer cell preparation followed by 0.1 ml of 0.1 M NaOH was added. It was allowed to stand for twenty minutes to reduce any keto acid and added 0.1 ml of 2.5 mg/ml sodium pyuvate solution. Extinction was read for five minutes at intervals of 15-30 seconds at 25°C as described elsewhere [18]. One enzyme unit activity produced to decrease in absorbance $\Delta A = 0.001$ per minute at 340 nm. The enzyme expressed the intracellular leakage during phagocytosis of Kupffer cells.

Plasminogen activator measurement: The plasminogen activator was measured based upon plasminogen dependent lysis by Kupffer cells. For it, nunc plates were coated with 10 μl 125 Fibrinogen 2 x 10^6 cpm per ml 0.1 % solution after centrifuging it at 600 x g for 2 minutes, dried over night at 45°C and stored at room temperature. In wells on next day 200 μl 10% acid treated fetal calf serum in Kreb's Hansleit buffer for four hours mixed and 28 % radioactivity was solubilized during procedure. Again wells were washed with buffer and 100 μl test supernatant Kupffer cell lysate with 20 μl acid treated fetal calf serum as plasminogen added followed by incubation for 24 hours at 37°C. Released activity in 100 μl aliquotes counted in form of total amount of disposable ^{125}I fibrin determined by incubating wells with 0.2 % trypsin in TC 199. Average value 100 taken and plasminogen dependent lysis calculated in percentage [19].

Hexose Monophosphate Shunt activity: was measured by the method described elsewhere [20]. $^{[14\text{-}C]}$ glucose 1 μCi/dish was added alongwith paticles latex and erythrocytes as mentioned above experimental cultures and without particlesas control. After one hour, 2 ml cell free medium was transferred into wells of Warburg type reaction flasks containing a strip of filter paper with 100 μl 10% potassium hydroxide in central well. The flasks were closed and 0.2 ml sulphuric acid added from side arm. Later filter paper strip and 250 μl water used for washing well and transferred to scientillation vial for counting in Trisol for $^{[14\text{-}C]}CO_2$. The unit of hexose monophosphate shunt activation was expressed in $^{[14\text{-}C]}CO_2$ liberated in dpm from labeled glucose per minute per mg protein.

Results

The cell cultures contained zymosan (7:1 particle cell ratio); erythrocytes (5:1 particle cell ratio); amebic trophozoits (2:1 particle ratio), latex (20:1 particle cell ratio) and nitroimidazole was added 1 µM/10^6 cells**.**

The glucuronidase activity showed the enhanced enzyme activity trend in all additives but different enzyme enhancement for different additives. In control cells, the activity enhanced during 24-36 hours in incubation. The erythrocytes showed most vulnerability to Kupffer cells in initial 24 hours. Zymosan beads showed slow response to phagocytosis while latex remained inert to Kupffer cells. The nitroimidazole showed normal enzyme pattern. It was interesting that maximum rate of phagocytosis was observed during 24 and 36 hours except erythrocytes.

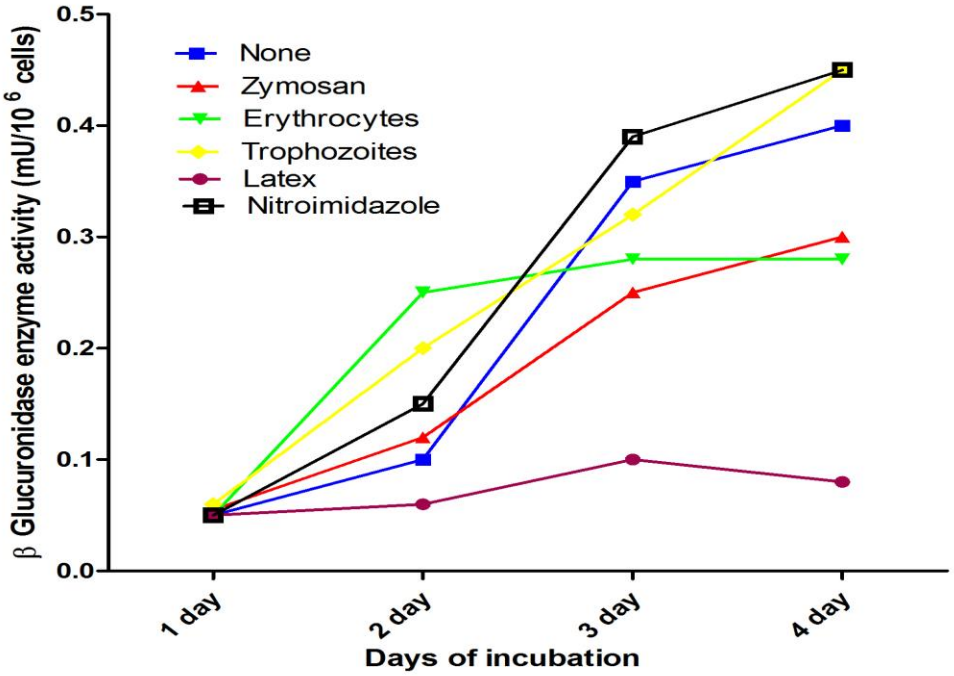

Figure 1: Phagocytic activity of Kupffer cells is shown as β glucuronidase enzyme activity of cells after adding zymosan, erythrocytes, entamoeba histolytica trophozoits, latex beads additives, and nitroimidazole as drug in cultures of Kupffer cells from liver biopsy samples. The enzyme activity was measured after 1, 2, 3, 4 days of incubation. Each point value represents the average of 3 readings of observations.

The lactate dehydrogenase enzyme activity showed the enhanced enzyme activity trend in all additives except latex additive but different enzyme enhancement for different additives. In control cells, the activity enhanced during 12-36 hours in incubation. The erythrocytes showed most vulnerability to Kupffer cells in initial 24 hours. Zymosan beads showed maximum response to phagocytosis while latex remained inert to Kupffer cells. The nitroimidazole showed inhibitory enzyme activity in Kupffer cells but after 24 hours the response was stimulatory to enzyme activity (delayed enhanced enzyme stimulation as a result phagocytosis). It was interesting that maximum rate of phagocytosis was observed during 48 hours.

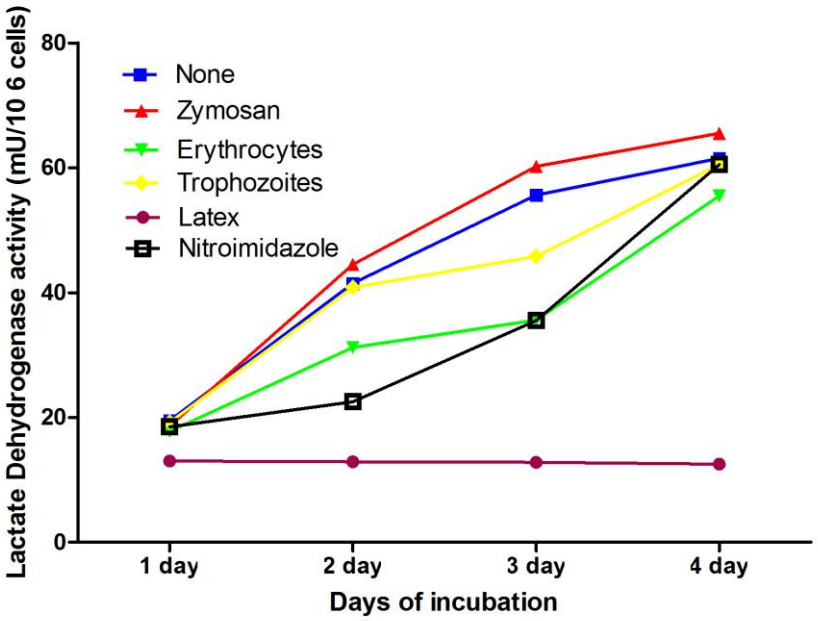

Figure 2: Phagocytic activity of Kupffer cells is shown as lactate dehydrogenase enzyme activity of cells after adding zymosan, erythrocytes, entamoeba histolytica trophozoits, latex beads additives, and nitroimidazole as drug in cultures of Kupffer cells from liver biopsy samples. The enzyme activity was measured after 1, 2, 3, 4 days of incubation. Each point value represents the average of 3 readings of observations.

The plasminogen activator induced lysis of Kupffer cells showed the enhanced plasmnogen activity only after 36 hours. Initially the activity trend in all additives was same and immune to any additive added. In control cells, the plasminogen activity remained unaltered while Kupffer cells showed immunity to zymosan and latex additives through out the incubation period. However Kupffer cells showed sharp enhancement of plasminogen activity after 36 hours during 36-48 hours in incubation. The trophozoits showed maximum effect on plasminogen activator in Kupffer cells after 36 hours.

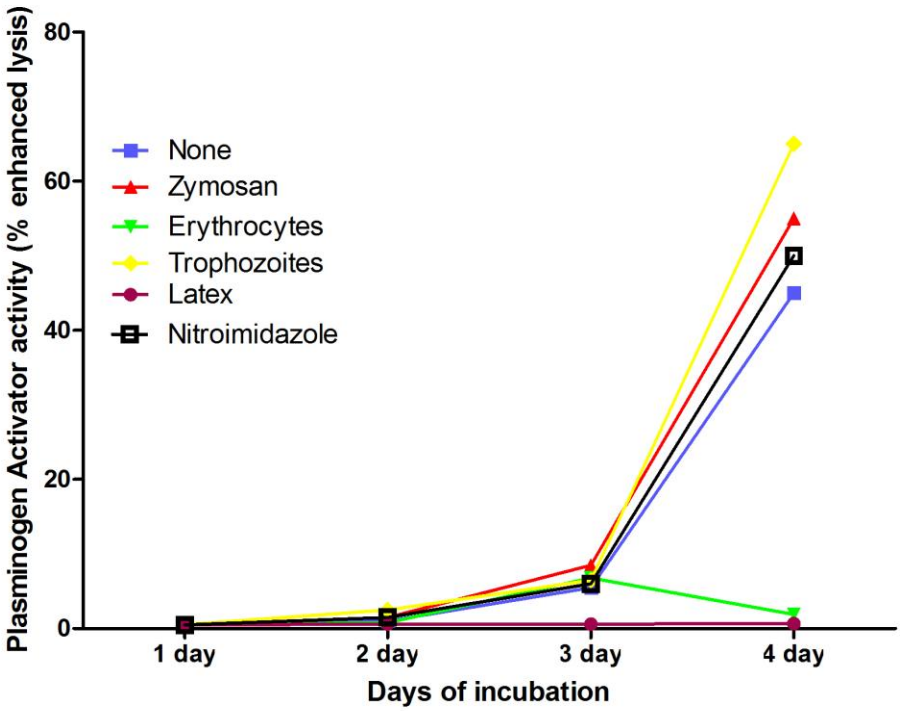

Figure 3: Phagocytic activity of Kupffer cells is shown as plasminogen activator activity of cells after adding zymosan, erythrocytes, entamoeba histolytica trophozoits, latex beads additives, and nitroimidazole as drug in cultures of Kupffer cells from liver biopsy samples. The enzyme activity was measured after 1, 2, 3, 4 days of incubation. Each point value represents the average of 3 readings of observations. Each point on graph represents the activity as extent of cell lysis in percent increase over basal value of 100.

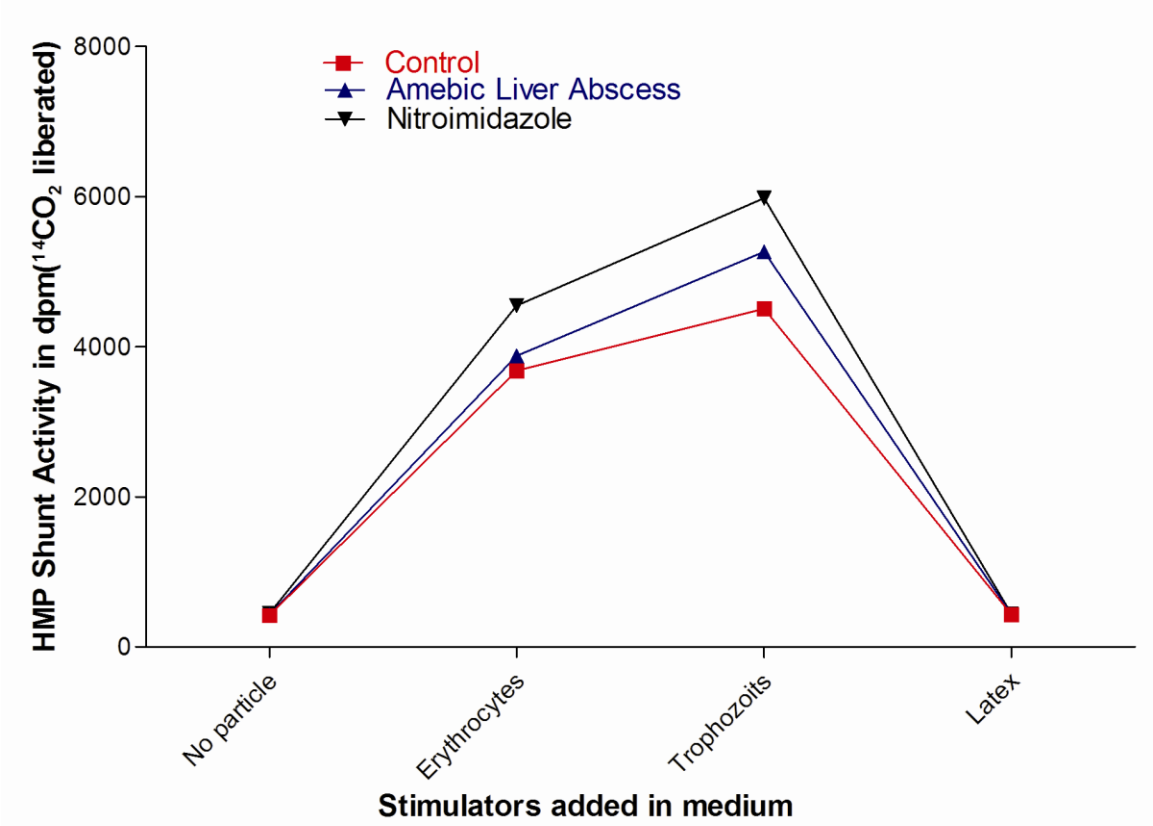

Figure 4: Hexose monophosphate shunt activity is shown in isolated Kupffer cells from ameba infected and nitroimidazole treated cells in presence of erythrocytes, trophozoits and latex additives. In 3.0 ml medium, 1 micro Curie 14C glucose was added and simultaneously particles added with Kupffer cells in ratio 5:1(particle:cell) for erythrocytes, 2:1 for amebic trophozoits and 40:1 for latex. After one hour, 2 ml of cell free medium soaked by filter paper wetted with 100 µL of 10% KOH in counter well which further was added with 200 µL 1.0 N H_2SO_4 and after 15 minutes filter paper strip elute in tritosol were measured for scientillation counts. The trophozoits showed maximum activity while inert latex beads did not show any change in activity.

Hexose monophosphate shunt activity in respiratory burst: The HMP shunt activity in control and triggered Kupffer cells was measured by [14-C]CO_2 release in dpm from labeled [14-C] glucose per minute per mg protein in presence of different effectors known as quenching the hexose monophosphates in glycolysis. The HMP shunt activity was similar with no difference (P value > 0.001) in control compared with triggered Kuffer cells with effector and inert latex particles did not alter the HMP shunt activity. The HMP shunt activity was significantly enhanced in triggered Kupffer cells in presence of trophozoites (P value < 0.001) but the HMP shunt enhancement was lesser in presence of erythrocytes. It indicated the dependance of HMP shunt activity on nature of effector either inert or surface active. The latex was inert, erythrocytes were nonvirulent and trophozoits were virulent as a result HMP shunt activity was enhanced in the order of latex < erythrocytes < trophozoits added in the medium of Kupffer cells.

DISCUSSION

Isolation of human Kupffer cells was developed by a modified pronase-collagenase enzymatic isolation and purity of Kupffer cells by using biomarker enzyme assay. Earlier reports used similar practice of enzymatic digestion and enzymatic assays as indicators of cell viability and yield [14, 15, 16]. A new rapid method was suitable for the isolation and fractionation of both rat and human Kupffer cells without the need of liver perfusion techniques. The study used rat livers or small human liver wedge biopsies obtained peroperatively and incubated with pronase under continuous pH registration. Kupffer cells were subsequently separated from other nonparenchymal cells by Nycodenz gradient centrifugation and purified by counterflow centrifugal elutriation [15]. Identification of Kupffer cells was achieved on the basis of ultrastructural analyses and immunophenotyping [15]. The fractionation of Kupffer cells gave good yield comparable with other studies [14, 15, 16]

Kupffer cell phagocytic activation in isolated Kupffer cells from human livers added with amoebic trophozoits, was elevated 2-3 fold over normal Kupffer cells. Kupffer cells incubated with zymosan and trophozoits exhibited maximum phagocytic activity while with latex and sheep erythrocytes these exhibited minimum and insignificantly increased activity respectively. Increased phagocytic activity was time dependent which was exhibited by β glucuronidase, lactate dehydrogenase and plasminogen activator activity. However, the leaked out LDH activity represented the probable phagocytic activity of Kupffer cells relationship with time. Nitroimidazole addition in incubation medium perhaps showed the minimizing amoebic virulence [17]. Earlier lysosomal enzyme release with increased LDH cellular levels has been reported [18]. Plasminogen activator induction has been reported dependent on de novo enzyme synthesis in stimulated macrophage by endotoxins and asbestos [19]. Phagocytosis of zymosan and sheep erythrocytes triggered the immediate release of β glucuronidase, stimulated synthesis of cellular lactate dehydrogenase and induced delayed production with secretion of plasminogen activator. The effect of nitroimidazole and latex were minimum. Similar observations were reported in earlier study [11, 20, 21]. Perhaps nitroimidazole was biotransformed into water soluble products and cleared by drug metabolizing enzyme system of Kupffer cells. Latex particles being inert confirmed the independence of Kupffer cell activation from intracellular fate of ingested material. Amoebic trophozoits with their toxins or specific membrane surface signals stimulate Kupffer cells for phagocytosis resulting early release of lysosomal hydrolases than the release by other particles [22, 23]. However, increased lysosomal enzymes in Kupffer cells incubated with amoebic trophozoits were reported as a consequence of phagocytic activation [23]. Recently, these phagocytic activation changes due to nitroimidazole have been reported as associated with prostaglandin synthesis and their release [24].

Although the clearance and distribution of ligand molecules in circulation represent the function of hepatic sinusoidal cells, these mechanisms revealed a network that is more intricate than would at first seem, since several receptors are common to not only one type of cell, but also to two or three types of cells in the liver. In the case of latex particles in which their uptake by a particular cell type seems to be determined by their size, sinusoidal endothelial cells are able to internalize particles up to 0.23 microns under physiologic

conditions, in vivo, and larger particles are taken up by Kupffer cells. However, when the phagocytic function of Kupffer cells were impaired by frog virus or alcohol, the endothelial cells were reported to take up particles larger than 1 micron in diameter after the injection of an excess amount of latex particles. Endothelial cells would thus constitute a second line of defense in the liver in that they remove foreign materials from the blood when Kupffer cell phagocytic function is totally disturbed [25]. This potential role may not, however, be fully expressed under physiologic conditions when Kupffer cells are active in clearing foreign substance from the circulation. The functions of liver sinusoidal cells are varied and complex and these cells can be regarded as "a sinusoidal cell unit." This cellular interaction must be taken into account for any quantitative analysis [8].

Hexose monophosphate shunt activation was enhanced during respiratory burst in isolated Kupffer cells in amoebic trophozoits and nitroimidazole added cultures. In the cultures added with trophozoits and nitroimidazole both, Kupffer cells exhibited significantly elevated HMP shunt activation. The HMP shunt activity was highly elevated in all groups cultures incubated with sheep erythrocytes and amoebic trophozoits while cells with latex particles did not show any activation. Perhaps latex particles being biologically inert could not change the cellular sinusoidal function activity. Sheep erythrocytes and trophozoits perhaps stimulated the glucose metabolism via hexose monophosphate pathway but latex particles were phagocytosed without altered shunt activity. It suggested that HMP shunt may be a triggering event in Kupffer cell activation dependent not upon intracelllular fate of ingested material or particles [26]. The trophozoits added Kupffer cells showed direct interaction with increased glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzyme activities as described as a result of enhanced hexose monophosphate shunt activity. Earlier zymosan stimulated and iodoacetate additives inhibited Kupffer cell glucose 6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities have been reported [27]. The hexose monophosphate shunt activity measured in terms of $^{[14\text{-}C]}$ glucose conversion into $^{[14\text{-}C]}CO_2$ has been described as inflammation elicited macrophage response [20].

References

1. Winwood PJ, Arthur MJ. (1993) Kupffer cells: their activation and role in animal models of liver injury and human liver disease. Semin. Liver Dis. 13(1):50-9.

2. Virk KJ, Ganguly NK, Dilawari JB, Mahajan RC (1989) Role of lysosomal enzymes in tissue damage during hepatic amoebiasis. An experimental study. Liver. 9 (6):338-45.

3. Nakashima H, Kinoshita M, Nakashima M, Habu Y, Shono S, Uchida T, Shinomiya N, Seki S. (1998) Superoxide produced by Kupffer cells is an essential effector in concanavalin A-induced hepatitis in mice. Hepatology. 48(6):1979-88.

4. Decker K. (1998) The response of liver macrophages to inflammatory stimulation. Keio J Med. 47(1):1-9.

5. Bilzer M, Roggel F, Gerbes AL. (2006) Role of Kupffer cells in host defense and liver disease. Liver Int. 26(10):1175-86.
6. Niimoto, S, Shimada H, Nakagawara G. (1990) Phagocytic activity of rat Kupffer cells--the measurement method in vitro and the effect of hepatocytes upon the phagocytic activity. Nippon Shokakibyo Gakkai Zasshi. 87(12):2614-22.

7. Shiratori Y, Tananka M, Kawase T, Shiina S, Komatsu Y, Omata M. (1993) Quantification of sinusoidal cell function in vivo. Semin Liver Dis 13(1):39-49.

8. Kamps JA, Morselt HW, Scherphof GL.(1999) Uptake of liposomes containing phosphatidylserine by liver cells in vivo and by sinusoidal liver cells in primary culture: in vivo-in vitro differences. Biochem Biophys Res Commun. 256(1):57-62.

9. Shiratori Y, Jin'nai H, Teraoka H, Matano S, Matsumoto K, Kamii K, Tanaka M, Okano K. (1989) Phagocytic properties of hepatic endothelial cells and splenic macrophages compensating for a decreased phagocytic function of Kupffer cells in the chronically ethanol-fed rats. Exp Cell Biol. 57(6):300-312.

10. Reske SN, Vyska K, Feinendegen LE. (1981) In vivo assessment of phagocytic properties of Kupffer cells. J Nucl Med. 22(5):405-10.

11. Dan C, Wake K.(1985) Modes of endocytosis of latex particles in sinusoidal endothelial and Kupffer cells of normal and perfused rat liver. Exp Cell Res. 158(1):75-85.

12. Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA.(2003) Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology. 37(5):1043-55.

13. Jayawant SS, Feldman SR, Camacho FT, Yentzer B, Balkrishnan R. (2008) Prescription refills and healthcare costs associated with topical metronidazole in Medicaid enrolled patients with rosacea. J Dermatolog Treat. 19(5):267-73.

14. Alabraba EB, Curbishley SM, Lai WK, Wigmore SJ, Adams DH, Afford SC. (2007) A new approach and culture of human Kupffer cells. J Immunological Met. 326(1-2):139-144.

15. Olynyk JK, Clarke SL. (1998) Isolation and primary culture of rat Kupffer cells. J Gastroenterol Hepatol. 13(8):842-5.

16. Kirn A, Steffan AM, Bingen A, Cinqualbre J, Gendrault JL.(1980) Isolation and culture of human Kupffer cells. C R Seances Acad Sci D. 291(2):249-51

17. Kausalya S, Kaur S, Malla N, Ganguly NK, Mahajan RC.(1996) Microbicidal mechanisms of liver macrophages in experimental visceral leishmaniasis. APMIS. 104(3):171-5.

18. Griffini P, Vigorelli E, Bertone V, Freitas I, Van Noorden CJ. (1994) Quantitative comparison between the gel-film and polyvinyl alcohol methods for dehydrogenase histochemistry reveals different intercellular distribution patterns of glucose-6-phosphate and lactate dehydrogenases in mouse liver. Histochem J. 26(6):480-6.

19. Nagaoka MR, Kouyoumdjian M, Borges DR.(2003) Hepatic clearance of tissue-type plasminogen activator and plasma kallikrein in experimental liver fibrosis. Liver Int. 23(6):476-83.

20. Kuttner RE, Schumer W, Apantaku FO. (1982) Effect of endotoxin and glucocorticoid pretreatment on hexose monophosphate shunt activity in rat liver. Circ Shock. 9(1):37-45.

21. Bhatnagar R, Schirmer R, Ernst M, Decker K. (1981) Superoxide release by zymosan-stimulated rat Kupffer cells in vitro. Eur J Biochem. 119(1):171-5.

22. Graham JM. Or Virk KJ, Ganguly NK, Prasad RN, Dilawari JB, Mahajan RC. (1989) Liver. 9(6):338-45.

23. Grahmm JM. (2001) Isolation of lysosomes from tissues and cells by differential and density gradient centrifugation. Curr Protoc Cell Biol. Chapter 3: Unit 3.6. John Wiley & Sons, Inc.

24. Spolarics Z, Wu JX. (1997) Tumor necrosis factor alpha augments the expression of glucose-6-phosphate dehydrogenase in rat hepatic endothelial and Kupffer cells. Life Sci. 60(8):565-71

25. Schorlemmer HU, Kurrle R, Schleyerbach R, Bartlett RR.(1999) Generation of O_2-radicals in macrophages can be inhibited in vitro and in vivo by derivatives of leflunomide's primary metabolite. Inflamm Res. 48 Suppl 2:S117-8

26. Knook DL, Sleyster EC, Teutsch HF.(1980) High activity of glucose-6-phosphate dehydrogenase in Kupffer cells isolated from rat liver. Histochemistry. 69(2):211-6.

27. Heuff G, Meyer S, Beelen RH.(1994) Isolation of rat and human Kupffer cells by a modified enzymatic assay. J Immunol Methods. 174(1-2):61-5.