



Isolation and characterization of murine Kupffer cells and splenic macrophages

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Abstract

A method is described using counterflow centrifugation elutriation to isolate macrophages from murine liver and spleen. In this study three, size fractionated, macrophage populations were collected. Isolation resulted in a high yield of pure Kupffer cells (total of 10×10^6 /g liver) and enrichment of splenic macrophages to 20%. In addition to standard methods such as non-specific esterase staining, the isolated macrophages were also characterized by flow cytometry using specific monoclonal antibodies. In addition, a rapid flow cytometry method was introduced to determine the percentage of macrophages based on autofluorescence. A strong correlation was found between the percentages of macrophages found by non-specific esterase staining and autofluorescence. Functional tests revealed differences between the isolated macrophages in terms of tumor necrosis factor- α (TNF- α) production, oxygen metabolism and the production of nitric oxide. However, no significant differences in phagocytic activity was observed between the fractions. After two weeks of culture without the addition of antibiotics the cells still exhibited the above mentioned functions.

Keywords: Kupffer cell; Splenic macrophage; Isolation; Elutriation; Flow cytometry

1. Introduction

Isolation of Kupffer cells (KC) from mice by counterflow centrifugation elutriation (CCE) was de-

scribed in 1977 by Knook and Sleyster (1977). More recently the purification of KC from mice, using the same technique has been reported by other workers (Janousek et al., 1993). Macrophages isolated under these conditions prompted the examination of functional characteristics *in vitro*, and provide excellent starting material. Moreover, several methods have been described using CCE for the isolation of KC from rat liver. Some of these methods are widely used and provide researchers with a fairly pure, though heterogeneous population of KC.

The isolation of splenic macrophages is far more difficult. The spleen possess different types of macrophages: the marginal zone macrophages, macrophages of the red pulp, the peri-arteriolar sheet,

Abbreviations: CCE, counterflow centrifugation elutriation; NSE, non-specific esterase staining; AF, autofluorescence; KC, Kupffer cells; TNF- α , tumor necrosis factor- α ; ROI, reactive oxygen intermediates; RNI, reactive nitrogen intermediates; DMEM, Dulbecco's modified Eagle medium; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; WB, working buffer; MoAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PI, propidium iodide; PMA, phorbol myristate acetate; cpm, counts per minute; NO, nitric oxide.

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and the marginal metallophils, all of which possess different characteristics (Humphrey and Grennan, 1981). Relatively few methods have been described for the isolation of splenic macrophages (Buckley et al., 1984; Ogle et al., 1994; Thompson et al., 1983). Due to the size of the organ, and the percentage of macrophages in the spleen, the collection of reasonable numbers of macrophages from the murine spleen is not easy.

It is clear that the spleen comprises different types of macrophages. However, the findings of others suggest that in the rat also the KC population is heterogeneous (Hoedemakers et al., 1993). Our own studies on murine liver and spleen by flow cytometry have indicated different activities of macrophages in these organs, which is in accordance with the findings of other groups working with rat liver (Daemen et al., 1989, 1991; Hardonk et al., 1989; Itoh et al., 1992).

Here we describe a method using CCE for the isolation of macrophages from mouse liver and spleen. The tissue macrophages were isolated under sterile conditions using an autoclavable elutriation rotor, which facilitates culture without the use of antibiotics. The cells were characterized by flow cytometry using monoclonal antibodies which differentiate between the macrophages and other cells, and between the different types of macrophages. Autofluorescence was used as an indicator of the percentage of macrophages in the cell populations, and showed a high correlation with the standard non-specific esterase method. In addition, the cells were cultured under more physiological non-adherent conditions, without the addition of antibiotics.

2. Materials and methods

2.1. Animals

Specified pathogen-free, 13–15-week-old male C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, Netherlands). The experimental design and animal care were performed according to institutional guidelines.

2.2. KC isolation

Mice were anesthetized i.p. with Nembutal (Sanofi, Paris, France). A ventral midline incision exposed the peritoneal cavity, and a sterile 24-gauge Teflon cannula (introcann-w. 24G $\frac{3}{4}$, Braun Melsungen, Melsungen, Germany) was inserted into the vena porta. The liver was preperfused in situ with calcium-free Hanks' buffered salt solution (Ca²⁺-free HBSS low endotoxin) (HyClone Laboratories, Logan, UT, USA) at pH 7.4 and 30°C, allowing the blood to flow from an incision in the vena cava inferior. After 2 min the wash solution was replaced by collagenase A solution (0.163 U/ml in DMEM high glucose) (Boehringer Mannheim, Mannheim, Germany), and the liver was perfused at a flow rate of 5 ml/min for 30 s at 35°C. After perfusion the liver was removed from the mouse and gently dispersed. Liver suspensions from eight mice were pooled and incubated under continuous agitation at 35°C for 10 min in 40 ml collagenase solution as was used for the perfusion in 50 ml polypropylene tubes. Hereafter all experiments were performed at 4°C. The liver cell suspension was filtered through a nylon gauze (212 μ m) and taken up in 50 ml HBSS containing 0.3% BSA (fraction V, low endotoxin, Sigma, St. Louis, MO, USA) and 2 μ g/ml DNase (low endotoxin, sterile, Boehringer Mannheim, Mannheim, Germany), and centrifuged at 400 \times g for 15 min. This buffer is subsequently referred to as working buffer (WB). The pellet was taken up in 15.6 ml WB and mixed with 21 ml Nycoprep (low endotoxin, sterile Nycodenz solution with a density of 1.150 g/ml and osmolarity of 290 mosM, Nycomed Pharma, Oslo, Norway). The mixtures were transferred to three 15 ml polypropylene tubes, and 1 ml WB was layered on top of the mixture. Red blood cells and debris was sedimented by centrifugation at 1500 \times g over 15 min. The cells in the interphase, which were mostly sinusoidal cells, were suspended in 50 ml WB and washed twice to remove Nycodenz by centrifugation at 400 \times g for 15 min. The pellet was taken up in 5 ml WB and the cells further separated into various fractions by CCE using the autoclavable Beckman JE-5.0 elutriator rotor supplied with a Sanderson chamber (Beckman Instruments, Palo Alto, CA, USA). The cell suspension was introduced into the elutriator at a flow rate of 12

ml WB per min, and the elutriator speed set to 3200 rpm. DNase was added to flow buffer (2 mg/l in HBSS/0.3% BSA) to prevent cell clumping in the elutriator chamber. Fractions of 100 ml were collected at flow rates of 12, 18, 26, 32 and 50 ml/min.

2.3. Isolation of splenic macrophages

Spleens were excised from anesthetized mice (see KC isolation) and spliced once with forceps. Organs from 15 mice were incubated under continuous agitation for 20 min at 35°C in 20 ml collagenase A solution (0.163 U/ml in HBSS). Thereafter the digest was gently pushed through a nylon gauze (88 μm), and the cell suspension collected in 50 ml WB containing 0.5 IU heparin. The cells were pelleted by centrifugation at $400 \times g$ for 15 min, resuspended in 5.5 ml WB and mixed with 7 ml Nycodenz. To prevent the cells in the interface from dehydration 1 ml WB was layered on top. The gradient was centrifuged for 15 min at $1500 \times g$. The interface cells were transferred to a 50 ml polypropylene tube containing WB and washed twice by centrifugation at $400 \times g$ for 15 min. The pelleted cells were resuspended in 5 ml WB and introduced into the elutriator at a flow rate of 12 ml WB per min, and the rotor speed set at 3200 rpm. Fractions between 100 and 400 ml were collected at flow rates of 12, 18, 24, 36 and 50 ml/min.

2.4. Culture of isolated KC and splenic macrophages

Isolated macrophages from fraction 3, 4, and 5 of the liver and spleen were brought to a density of 1.0×10^6 cells/ml in DMEM high glucose culture medium (Gibco, Life Technologies, Gaithersburg, MD), supplemented with 15% FCS (Gibco). The macrophages could be cultured in 5% CO_2 for up to 2 weeks at 37°C and in 96 well plates (MatTek, Ashland, MA, USA), using 150 000 cells per well, with continuous shaking at 110 rpm, or in plastic 96 well Falcon culture plates (Becton Dickinson, San Jose, CA).

2.5. Characterization of isolated cell populations

Flow cytometric analysis was performed using of rat anti-mouse monoclonal antibodies (MoAb) as

hybridoma culture supernatants. F4/80 (murine M ϕ) (Austyn and Gordon, 1980), MOMA-2 (murine M ϕ) (Kraal et al., 1987), RA3-6B2 (B cells) (Coffman, 1982), KT3 (T cells) (Tomonari, 1988), 30G12 (general leukocyte marker) (Ledbetter and Herzenberg, 1979), and Y3 a non-reacting negative control. All hybridoma supernatants were kindly provided by P. Leenen and J. Voerman (Department of Immunology, Erasmus University, Rotterdam, Netherlands). 25 μl cell suspension was mixed with 25 μl hybridoma supernatant and incubated at room temperature for 10 min. Cell suspensions were washed three times with PBS containing 5% FCS and 20 mM sodium azide. Thereafter cells were incubated with an FITC-conjugated rabbit anti-rat antibody, (F(ab')₂ fragment). Free conjugate was removed by washing as described above. The cell suspensions were examined on a FACScan and evaluated with Consort 30 software (Becton Dickinson, San Jose, CA).

2.6. Determination of cell viability

Cells were incubated after isolation with 50 $\mu\text{g/ml}$ propidium iodide (PI) (Sigma, St. Louis, MO, USA). Dead cells take up PI which intercalates with the DNA resulting in red fluorescent cells. The percentage of dead cells was determined by flow cytometry.

2.7. Autofluorescence (AF) as a quantitative marker of macrophage percentages in cell populations

Macrophages are cells with a pronounced autofluorescent activity (Thorell, 1981). The percentage of autofluorescent cells (AF⁺) was evaluated by flow cytometry in the green emission range (F11) (excitation 488 nm, emission 520 nm).

2.8. Non-specific esterase (NSE) activity of the purified cell fractions

The purity of the cell fractions was verified on cytospin preparations after staining for NSE activity (Stroben, 1994). Briefly, cells were fixed with ice-cold formalin-acetone and incubated with pararosaniline/a-naphthyl butyrate. Cells were then counterstained with Gills hematoxylin and examined microscopically.

2.9. Quantitation of production of reactive oxygen (ROI) and nitrogen (RNI) intermediates

Oxygen metabolism was examined by chemiluminescence 4 h after isolation. Briefly, 1×10^6 cells in 1 ml were mixed with $100 \mu\text{l}$ 5×10^{-3} M luminol (Sigma, St. Louis, MO, USA), and triggered with $100 \mu\text{l}$ 5×10^{-5} M phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA). Chemiluminescence was measured using an AutoLumat LB953 (EG & G Berthold, Wildbad, Germany). Nitrite production was measured using Griess reagents as reported by Stuehr and Nathan (1989) 6 days after isolation. Briefly, 1.5×10^5 macrophages in $150 \mu\text{l}$ DMEM (without phenol red) supplemented with 10% FCS, were exposed to heat killed *Klebsiella pneumoniae* for 0, 1, 2 or 4 h, and then $150 \mu\text{l}$ of Griess reagents were added. Absorption at 570 nm was determined and compared with a standard curve prepared at the same time using known concentrations of sodium nitrite.

2.10. Assessment of TNF- α production by KC

After 6 days of culture KC were exposed to heat killed *Klebsiella pneumoniae* using a ratio of 15 bacteria per macrophage for 4 h. TNF- α levels were determined in the culture medium using the WEHI 164 bioassay (Espevik and Nissen-Meyer, 1986).

2.11. Phagocytic assay

The microorganism used for phagocytosis was *Candida albicans*, grown for 24 h at 37°C in Sabouraud maltose broth (Oxoid, Basingstoke, Eng-

land), washed three times with PBS and preserved on ice. KC fractions were cultured in 96 well plates at 200 000 cells per well for 6 days in 200 μl DMEM supplemented with 15% FCS. After that cells were incubated with *C. albicans* using a ratio 50 microorganisms per macrophage. Non phagocytosed organisms were removed by washing the monolayers with PBS, and phagocytosis was examined microscopically after Diff-Quick staining.

3. Results

3.1. Characterization of isolated KC

Directly after separation by CCE, cell fractions were examined with respect to total cell number and the percentages of different cell types. Fraction 1, collected at a flow rate of 12 ml/min, only contained debris and no cells are present in this fraction. Fraction 2 contained large numbers of endothelial cells and KC. Routinely around 80×10^6 KC were recovered in fraction 3 and around 20×10^6 KC were recovered in fraction 4, whereas 10×10^6 KC were collected in fraction 5 at 50 ml/min. A total of 10×10^7 KC were collected from eight mice. The viability of these cells, as assessed by propidium iodide exclusion after isolation, appeared to be between 85 and 95%.

As shown in Table 1, an increasing percentage of macrophages detected by NSE staining were collected by increasing flow rates: NSE positive cells increased from approximately 30% in the unseparated fraction to above 95% in fraction 5. These

Fig. 1. Kupffer cells isolated by counterflow centrifugation elutriation were stained on slides with Diff Quick (A) or non-specific esterase (B). Typical characteristics of the KC, membrane ruffling and large nucleus (N) are shown in the Diff Quick staining (original magnification $1000 \times$). Kupffer cells are stained brown in the non-specific esterase reaction. A sample was taken from fraction 2 to enhance the differences in non-specific esterase staining (original magnification $400 \times$).

Fig. 2. Splenic macrophages isolated by counterflow centrifugation elutriation were stained on slides with (left panel) Diff Quick (2–5, numbers correspond with isolated fractions) or (right panel) non-specific esterase staining of fraction 5 only (original magnification $200 \times$). Macrophages are characterised by brownish staining in the cytoplasm (arrows), whereas granulocytes (PMN) do not stain with the non-specific esterase reaction (original magnification $400 \times$).

Fig. 8. Phagocytosis of *Candida albicans* by Kupffer cells in fractions 3, 4 and 5. Kupffer cells in a monolayer were exposed to 20 microorganisms per macrophage for 10 min. Fixed monolayers were stained with Diff Quick and 400 cells were examined per monolayer. Phagocytosed microorganisms (arrow head) and nucleus of Kupffer cells (arrow) are visible (original magnification $200 \times$).

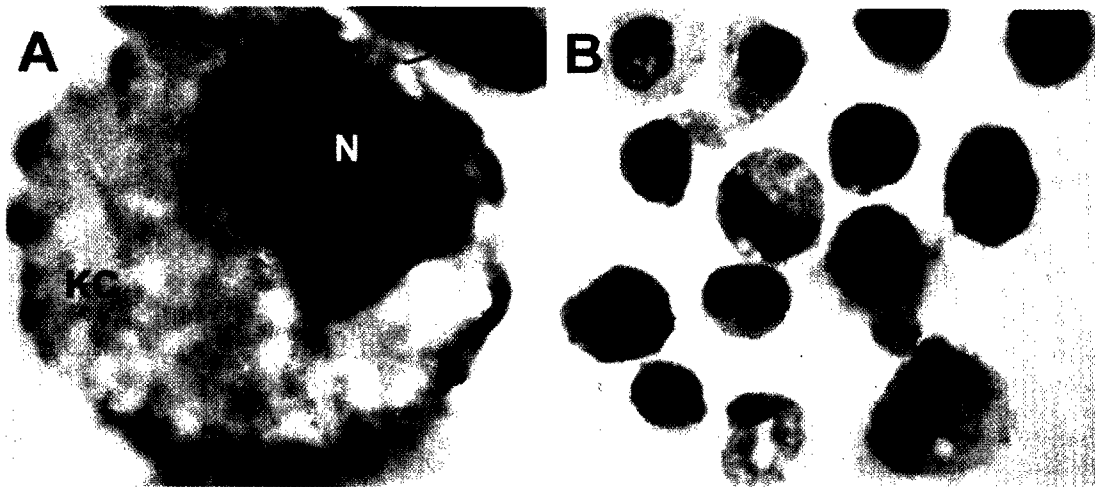


Fig. 1

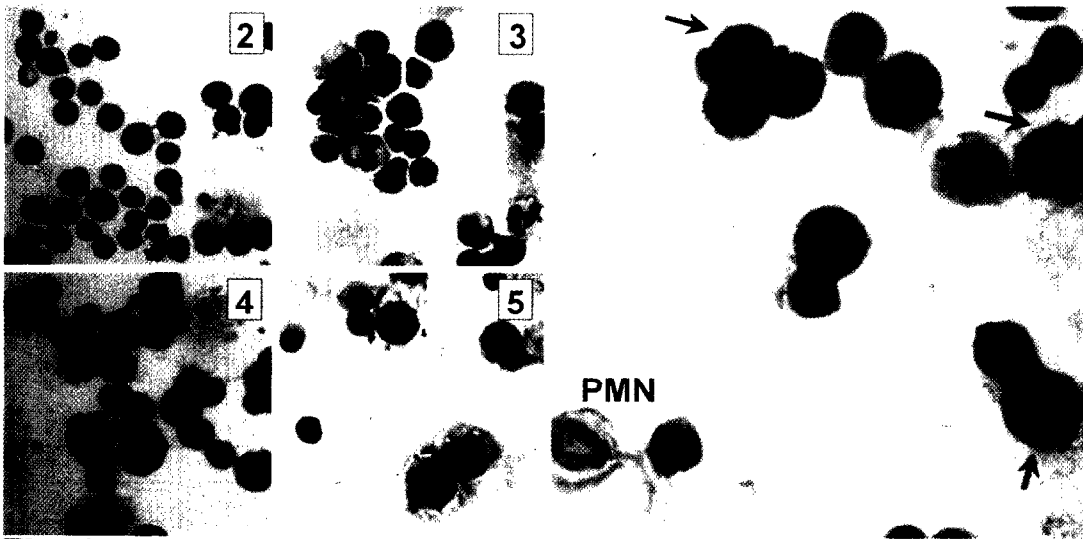


Fig. 2

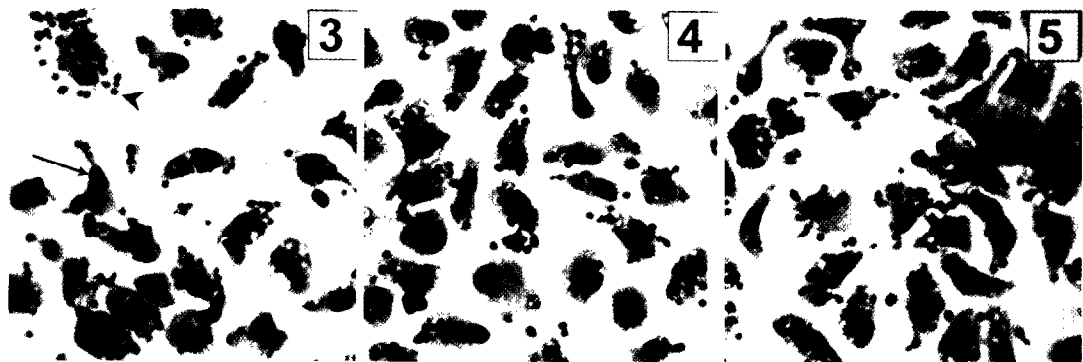


Fig. 8

Table 1
Characteristics of Kupffer cells isolated from murine liver by counterflow elutriation centrifugation (CCE)

	Kupffer cell fraction number			
	2	3	4	5
Fraction size (ml) ^a	100	100	100	100
Number of cells ($\times 10^6$)	57.3 \pm 17.2	79.9 \pm 22.3	21.4 \pm 6.4	10.5 \pm 3.3
% positive cells ^b				
NSE ^c	41.3 \pm 3.4	80.9 \pm 1.2	> 92.5	> 95
DQ	17.9 \pm 1.3	86.7 \pm 2.1	> 92.5	> 95
F4/80	20.9 \pm 8.1	50.2 \pm 8.1	56.1 \pm 5.7	47.3 \pm 3.7
MOMA-2	32.1 \pm 4.2	61.7 \pm 4.7	68.2 \pm 4.8	58.4 \pm 7.7
AF	51.9 \pm 9.7	80.0 \pm 1.0	88.9 \pm 4.4	90.5 \pm 3.6

The data represents the mean \pm 1 SD of at least six experiments.

^aFraction collected from the CCE.

^bPercentage of positive cells were determined for non-specific esterase activity and after Diff Quick staining on cell cytopins on slides by counting 400 cells. Percentage of F4/80 or MOMA-2 positive cells was determined by flow cytometry.

^cNSE: non-specific esterase; DQ: Diff Quick; F4/80 and MOMA-2: macrophage-specific monoclonal antibodies; AF: autofluorescence.

results were confirmed by Diff-Quick staining, which clearly showed the presence of the majority of the macrophages in the last three fractions, recognizable by their asymmetric nucleus, vacuolized cytoplasm and membrane ruffling (Fig. 1).

Flow cytometry was also used to quantify KC. Whereas only 50.2, 56.1 and 47.3% of the cells in fractions 3, 4 and 5 respectively were found to be

positive for F4/80, MOMA-2 recognized 61.7, 68.2 and 58.4% of the cells in the fractions 3, 4 and 5 when FITC conjugated antibodies were used (Table 1). Using AF to evaluate the macrophage content of the fractions, 51.9% of the cells appeared to be positive in fraction 2, whereas fractions 3, 4 and 5 contained 80.0, 88.9 and 90.5% AF⁺ cells respectively.

Table 2
Characteristics of macrophages isolated from murine spleen by counterflow elutriation centrifugation (CCE)

	Spleen cell fraction number			
	2	3	4	5
Fraction size (ml) ^a	100	400	200	200
Number of cells ($\times 10^6$)	952 \pm 188	174 \pm 83.6	78.7 \pm 34.7	17.5 \pm 5.0
% positive cells ^b				
NSE ^c	< 1	4.0 \pm 0.3	17.6 \pm 1.8	18.3 \pm 1.9
DQ	< 1	10.3 \pm 1.1	11.5 \pm 1.3	14.0 \pm 1.5
F4/80	2.2 \pm 0.4	11.4 \pm 4.7	21.8 \pm 4.5	21.9 \pm 6.4
MOMA-2	1.8 \pm 0.4	6.4 \pm 1.4	19.6 \pm 1.3	19.4 \pm 6.3
AF	0.9 \pm 0.6	4.9 \pm 3.3	12.8 \pm 6.5	14.3 \pm 3.7
KT3	25.7 \pm 5.8	16.0 \pm 0.2	8.6 \pm 0.1	21.5 \pm 0.4
RA3-6B2	46.4 \pm 6.4	41.9 \pm 0.1	33.3 \pm 0.4	34.7 \pm 0.7
RB6-8C5	3.9 \pm 1.8	19.1 \pm 7.8	21.8 \pm 4.1	21.6 \pm 3.2

The data represents the mean \pm 1 SD of at least six experiments.

^aFraction collected from the CCE.

^bPercentage of positive cells were determined for non-specific esterase activity and after Diff Quick staining on cell cytopins on slides by counting 400 cells. Percentage of F4/80, MOMA-2, KT3, RA3-6B2, and RB6-8C5 positive cells was determined by flow cytometry.

^cNSE: non-specific esterase; DQ: Diff Quick; F4/80 and MOMA-2: macrophage-specific monoclonal antibodies; AF: autofluorescence; KT3: T cell; RA3-6B2: B cell-, and RB6-8C5: granulocyte-specific monoclonal antibodies.

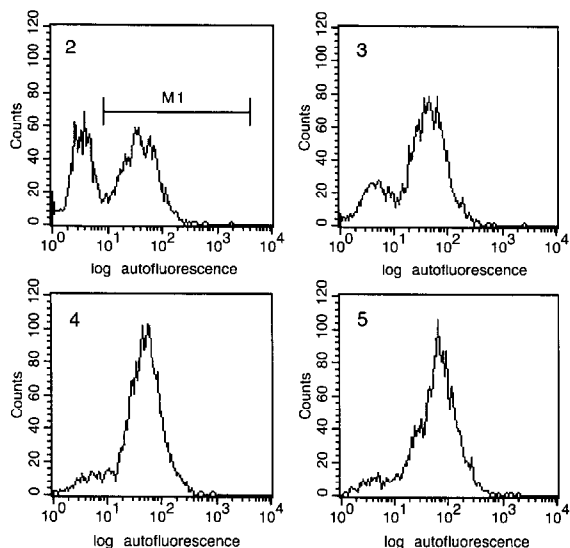


Fig. 3. Analysis of Kupffer cell fractions 2-5, collected by counterflow centrifugation elutriation, by flow cytometry using autofluorescence to positively identify macrophages. Cells in the area M1 are AF⁺. The graph numbers correspond to the fraction numbers collected. The graphs are representative of at least six experiments.

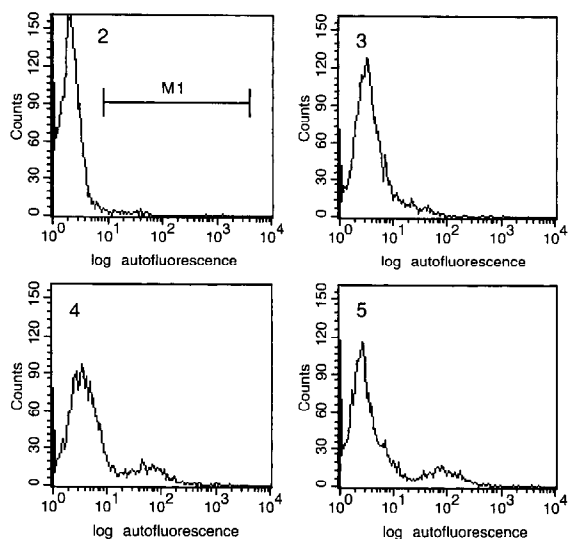


Fig. 4. Analysis of spleen macrophage fractions 2-5, collected by counterflow centrifugation elutriation, by flow cytometry using autofluorescence to positively identify macrophages. Cells in the area M1 are AF⁺. The graph numbers correspond to the fraction numbers collected. The graphs are representative of at least six experiments

3.2. Characterization of isolated splenic macrophages

Fraction 1, collected at 12 ml/min did not contain any cells. Splenic macrophages were present in

fractions 3, 4 and 5, as confirmed by cytospin preparations followed by Diff-Quick or NSE staining (Fig. 2). Less than 1% of the unseparated cells were found to be NSE positive. Flow cytometric analysis revealed that 11.4, 21.8 and 21.9% of the cells were

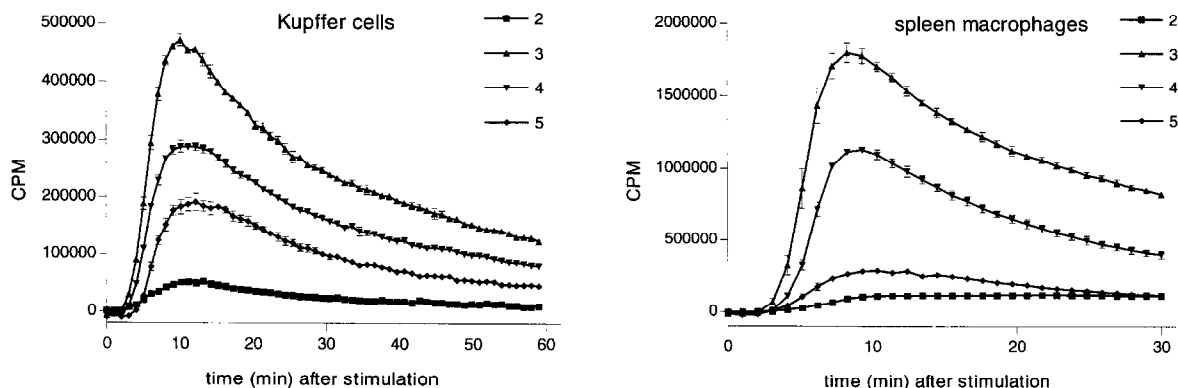


Fig. 5. Chemiluminescence of Kupffer cell fractions 2-5 and spleen macrophage fractions 2-5 in suspension, collected by counterflow centrifugation elutriation, with PMA used as trigger. Chemiluminescence of unstimulated cells was below the threshold of detection. The data represent the mean \pm 1 SD of three experiments.

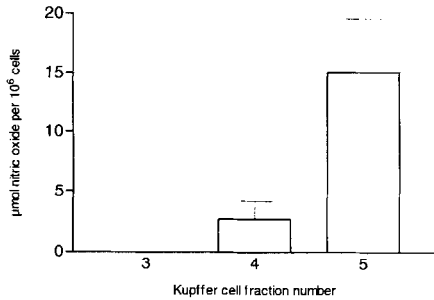


Fig. 6. Production of nitric oxide by Kupffer cell fractions 3, 4 and 5 4 h after stimulation with heat killed *Klebsiella pneumoniae*. Production of NO by unstimulated cells was below the detection level. The data represents the mean \pm 1 SD of three experiments.

positive for F4/80 whereas 6.4, 19.6 and 19.4% of the cells stained with MOMA-2 in fractions 3, 4 and 5 respectively (Table 2). The percentages of AF⁺ cells determined by flow cytometry in spleen fractions 2, 3, 4 and 5 were < 1, 4.9, 21.8 and 21.6% respectively.

The spleen is mostly composed of T and B cells. We therefore examined the percentage T cells (KT3)

and B cells (RA3-6B2) in the different fractions. Neither cell type was removed effectively from the cell populations by CCE (Table 2). The number of T cells in the various fractions did not differ significantly, whereas the percentage of B cells decreased from 63.1% before CCE to 44.3% in fraction 3. Granulocytes (RB6-8C5) also exhibit phagocytic activity and produce oxygen intermediates. Therefore, the percentage of these cells in the fractions was evaluated. It appeared that the percentage of granulocytes in the spleen cell fractions increased after CCE compared to the complete cell suspension (4.6% before and 20% granulocytes after CCE).

The percentages of cells positive for NSE and positive in the Diff-Quick staining in both the KC and splenic macrophage isolations appeared to correlate closely with the percentages of cells exhibiting AF activity. As can be seen in Table 1 the percentage of AF⁺ cells increased from 51.9 to 90.5% between KC fractions 2 and 5. NSE positive cells increased from 41.3 to > 95% (correlation coefficient, r^2 0.998). A similar correlation was noted with splenic macrophages with an increase from 0.9

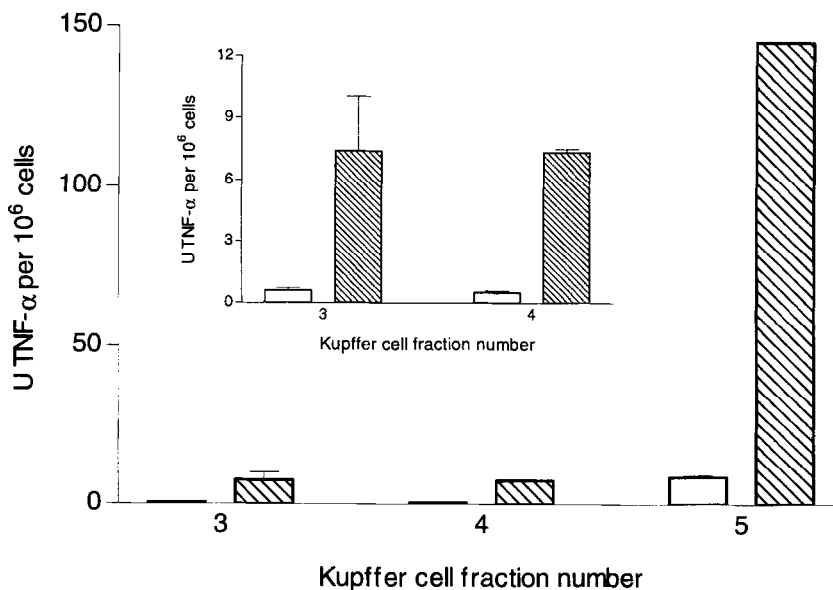


Fig. 7. Production of TNF- α by Kupffer cell Fractions 3, 4 and 5 12h after stimulation with heat killed *Klebsiella pneumoniae* (hatched bars) or with medium alone (open bars). The data represent the mean \pm 1 SD of three experiments.

to 14.3% for AF and < 1 to 18.3% for NSE (r^2 0.989). Figs. 3 and 4 illustrate how the percentage of AF⁺ cells (macrophages) can be determined by flow cytometry for both liver and spleen.

In our hands more macrophages could be isolated from liver and spleen after the addition of DNase and we did not find a deleterious effect of DNase on cell viability in contrast to others (Janousek et al., 1993). In the absence of DNase the isolation procedure resulted in cell clumping, a low yield, and especially for the splenic macrophages in very irreproducible results (Sanderson and Bird, 1977).

Cells were examined for oxygen metabolism in suspension after stimulation with PMA. Fig. 5 shows that KC fractions exhibited different levels of oxygen metabolism. Fraction 2 had little metabolic activity (53 000 peak cpm), whereas fraction 3 was most active (470 000 peak cpm). Oxygen metabolism decreased again in fractions 4 and 5 (respectively 290 000 and 190 000 peak cpm). This activity appeared to be superoxide dependent: in all fractions chemiluminescence was decreased by around 70% following the addition of superoxide dismutase (data not shown).

Oxygen metabolism in the spleen cell fractions was much higher compared to the KC cells (Fig. 5). Fraction 3 showed strong activity (1 800 000 peak cpm), which was decreased in fraction 4 (1 130 000 peak cpm). Fractions 2 and 5 were characterized by low oxygen metabolism compared to the other fractions (110 000 and 290 000 peak cpm respectively). Because of the impurity of the spleen cell fractions and the low yield of splenic macrophages further functional tests were performed only with KC.

The production of significant amounts of NO could only be measured in the culture supernatants of KC fraction 5 after stimulation with heat killed *Klebsiella pneumoniae*. KC in fraction 5 produced around 15 μmol NO per 10^6 cells, whereas there was no detectable NO production in supernatants of fraction 3 (Fig. 6).

KC purified by CCE and cultured for 6 days were assayed for TNF- α production. Fraction 5 appeared to produce high levels of TNF- α (150 U per 10^6 cells), whereas fractions 3 and 4 produced small but significant amounts (around 8 U TNF- α per 10^6 cells), after triggering with heat killed *Klebsiella pneumoniae* (Fig. 7).

All KC fractions exhibited strong phagocytic activity when exposed to *Candida albicans*. After 10 min of exposure almost 100% of the cells contained microorganisms. KC appeared to phagocytose around 20 microorganisms per macrophage when exposed for 10 min to 50 microorganisms per macrophage (Fig. 8). We observed a TNF- α and RNI production of approximately 60% after two weeks of culture, and all cells still phagocytosed *C. albicans* at that time point (data not shown).

4. Discussion

In most publications related to the isolation of resident macrophages from liver, KC are considered to be a single population (Heuff et al., 1993, 1994; Janousek et al., 1993; Knook and Sleyster, 1977; Ogle et al., 1994; Sanders and Soll, 1989). Only few groups have described the isolation of different fractions of macrophages from the liver of the rat (Daemen et al., 1989, 1991; Hardonk et al., 1989; Itoh et al., 1992; Hoedemakers et al., 1993) and from spleen (Thompson et al., 1983). Cell populations of rat liver macrophages have been functionally examined with emphasis on tumor cytotoxicity and response to immunomodulator treatment (Daemen et al., 1989, 1991; Hardonk et al., 1989; Hoedemakers et al., 1993). The findings of others in rats and our own (unpublished) observations in vivo in mice, led us to the isolation of subgroups of macrophages from liver and spleen in order to study the heterogeneity of mouse macrophages. In the present study a method is described in which murine KC and splenic macrophages are isolated and fractionated into three groups of macrophages depending on their size by CCE. It appeared that these cells not only differed in their size but were also characterized by a completely different range of activities.

Three fractions of KC were isolated with purities ranging from 81 to > 95% as estimated with NSE and this was confirmed by autofluorescence. However, lower percentages were found when specific MoAb were used (F4/80 50.2–56.1% and MOMA-2 61.0–68.2%). This discrepancy was most probably due to (1) the low expression of the surface markers of the macrophages and (2) to the specificity of the MoAb resulting in detection of only a subgroup of

the macrophages. Furthermore the phagocytosis of *C. albicans* suggested that the fractions contained large numbers of macrophages. The overall yield was around 10×10^7 cell per isolation, which means that around 1×10^7 KC were isolated per gram mouse liver. Fraction 3 contained small KC, fraction 5 contained large, and fraction 4 contained intermediate sized KC. The isolated KC in the different fractions exhibited different metabolic activities. The production of ROI by KC could not be explained by the presence of granulocytes, because such cells were not detected using our screening methods. However, these findings contrast with those of Lepay et al. (1985), who found KC to be deficient in production of ROI. It is possible that this group selectively isolated large KC which exhibit only very low ROI production.

Although fraction 3 cells showed strong oxygen metabolism, they produced only very small amounts of TNF- α or nitric oxide after stimulation. Fraction 4 was comparable to fraction 3, but fraction 5, which was composed of large KC, showed a significant production of TNF- α and nitric oxide. These results are comparable with those of Decker et al. (1989) and Ogle et al. (1994), suggesting that their KC population contained predominantly large KC. However, Shimauchi et al. (1993) reported that KC produced much lower amounts of TNF- α , which might be explained by a KC population composed of predominantly small or intermediate cells. We found that an improved separation could be obtained between the KC fractions when the rotor speed was set to 3200 rpm, compared to the generally used setting of 2400 rpm (unpublished observations). We also used this setting for splenic macrophage isolation.

The isolation of murine splenic macrophages proved far more difficult. Various fractions were collected by CCE and increasing percentages of macrophages from 4% up to 11.4%, 21.8%, and 21.9% respectively were obtained in the last three fractions as detected with MoAb F4/80. Flow cytometry using the MOMA-2 antibody yielded comparable percentages of 6.4, 19.6, and 19.4 respectively. These findings are in agreement with Buckley et al. (1984), who isolated one fraction from human spleen with 28% mononuclear phagocytes, but they differ from those of Thompson et al. (1983). In the latter study various fractions were isolated but the

percentage of macrophages ranged from 3 to 5.2%. In the spleen MoAb F4/80 predominantly recognizes red pulp macrophages, whereas MOMA-2 binds to (mature) white pulp macrophages, as well as to red pulp macrophages (review by Leenen et al., 1994).

Production of ROI by splenic macrophages in the different fractions was similar to that observed with the KC fractions. However, the interpretation of these results are hampered by the presence of granulocytes in the fractions, which exhibit strong oxygen metabolism. It appeared that the percentage of granulocytes in the last three fractions were practically the same, which suggests that the percentage of granulocytes does not account for the differences in ROI production. It is unclear whether the differences in metabolic activity in the three fractions were related to differences in the macrophages, or whether size separation of granulocytes also occurs resulting in granulocytes with different oxygen metabolism.

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