

# MASS ISOLATION AND CULTURE OF RAT KUPFFER CELLS

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The Kupffer cells (K cells)<sup>1</sup> of the liver may be the most important mononuclear phagocytes of the body. In accordance with this is the vast literature on the composition, structure, and function of the cell in vivo (1). However, some of the most important problems of K-cell function are still unresolved. This is among other things due to technical obstacles: the K cells represent only a small part of the liver, greatly outmassed as well as outnumbered by parenchymal cells, fibroblasts, and endothelial cells. Thus a K-cell preparation tends to be either heavily contaminated with other cell types, or if sufficiently purified, only available as a small fraction of the total cell number and therefore hardly representative. Even though there have been occasional reports on isolated cells in primary suspension (2, 3), and cultivation of small numbers of K cells (4-6) there has been so far no available high yield, high purity method for providing a representative population of K cells in tissue culture, allowing them to be studied under strictly defined in vitro conditions.

The advent of the technique of dispersing the liver by collagenase perfusion (7) followed by selective enzymatic digestion of parenchymal cells by pronase (8, 9) has opened the possibility of obtaining pure K-cell preparations for in vitro studies. This is the first in a series of reports on the establishment and characterization of mass culture of rat K cells.

## Material and Methods

*Animals.* Adult female Wistar rats, 170-200 g were obtained from Møllegaard Hansens Laboratories A/S, Ejby, Denmark. The rats were fed a standard pellet diet and water ad libitum.

*Chemicals.* Pronase (type 1) was obtained from Calbiochem, Los Angeles, Calif. Collagenase (type 1) and enzyme substrates were from Sigma Chemical Co., St. Louis, Mo.

*Preparation of Liver Cells.* Liver cell suspensions were prepared essentially as described earlier (10, 11). In summary, the liver was removed from the animal and perfused in vitro, first with Ca<sup>++</sup>-free Hanks' solution for 5 min and then with Hanks' solution containing 5 mM Ca<sup>++</sup> and 0.05% (wt/vol) collagenase. Vigorous bubbling of the perfusion medium with 95% O<sub>2</sub> and 5% CO<sub>2</sub> kept the pH at 7.45 and the Po<sub>2</sub> above 400 mm Hg throughout the procedure. The perfusion was stopped after 10 min, and a cell suspension was obtained by shaking the liver gently at 4°C in Hanks' solution without carbonate but with 20 mM HEPES buffer. Purified parenchymal cells were prepared by low

<sup>1</sup>Abbreviations used in this paper: HMEM, HEPES-buffered MEM; K cells, Kupffer cells; NBCS, newborn calf serum; NPC, nonparenchymal cells.

speed differential centrifugation (10). Nonparenchymal cells (NPC) were obtained essentially as described previously (9), by incubating portions of the initial cell suspension at 39°C in the presence of pronase. During this treatment parenchymal cells are selectively destroyed and pure NPC can be separated from the debris by centrifugation and repeated washing in Hanks' solution with HEPES buffer. The efficiency of the pronase digestion of parenchymal cells as a function of pH, initial cell concentration, and pronase concentration was studied, and the results indicated that digestion of parenchymal cells proceeds optimally at pH 7.5 in the presence of 0.1% pronase and with approximately  $2.5 \times 10^6$  parenchymal cells/ml present initially. All parenchymal cells are destroyed within an hour under these conditions.

*Biochemical Determinations.* Protein was determined according to the method of Lowry et al. (12) and DNA according to the method of Burton (13). Acid DNase (14) was determined at pH 5 with calf thymus DNA as substrate and with 0.15 M KCl present.  $\beta$ -glucuronidase was determined at pH 5 with phenolphthalein glucuronic acid as substrate (14). Cathepsin D was measured at pH 3.5 with bovine hemoglobin as substrate (15).

*Carbon Injections.* 0.1 ml of ink (Günther Wagner, Hannover, West Germany) was injected into the tail vein of the rats 1 or 2 h before liver perfusion. In some cases the injection was repeated 24 or 48 h later and 1–2 h before perfusion.

*Tissue Culture.* After the final washing, the cells were suspended at a concentration of  $0.5 \times 10^6$  cells/ml in minimal essential medium (MEM, Bio-cult, Glasgow, Scotland) with either 30% newborn calf serum (NBCS, Grand Island Biological Co., Grand Island, N. Y.), 5% rat serum, or 20% horse serum (GIBCO). All sera were first inactivated for complement at 56°C for 30 min. Portions of 1 ml were left to sediment in Linbro Tissue Culture plates with 16-mm troughs (FB-16-24-TC Linbro Chemical Co. Ltd., New Haven, Conn.) with or without fitted glass cover slips. For Fc receptor studies  $2 \times 10^6$  cells were seeded into 35 mm Falcon Plastic petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) fitted with 25 mm circular cover slips. After 20 h the dishes were washed with warm medium and 1 ml fresh medium with serum was added to each trough. All experiments were performed on K cells after 20–24 h in vitro culture. Some cultures were left for 1 wk or more for observation of the effect of long-term culture. The biochemical assays were performed after washing in saline and disintegration of the cells by freezing and thawing three times in distilled water. Mouse peritoneal macrophages were cultivated according to Cohn and Benson (16).

*Phagocytosis Studies.* Dow Latex particles of 1.1  $\mu$ m diameter were obtained from SERVA Entwicklungslabor, Heidelberg, West Germany. The stock solution was diluted with medium and added to the culture dishes in a concentration where each cell was in contact with at least 10 particles after sedimentation. The cells were then incubated with the particles for up to 3 h. At the end of the incubation the cultures were rinsed with warm medium, and fixed for morphological examination.

*Pinocytosis Studies.* Radioactive colloidal gold ( $^{198}\text{Au}$ ) was obtained from the Institutt for Atomenergi, Kjeller, Norway. An amount corresponding to 2.5  $\mu$ Ci was added to each culture trough at the beginning of the experiment. Equal amounts of radioactive material were added to troughs without cells to obtain background values of absorbed radioactivity. At the end of the desired periods of incubation, the cultures were rinsed thoroughly with warm medium and the cover slips were assayed for radioactivity with a  $\gamma$ -spectrophotometer (Nuclear Enterprises Ltd., Edinburgh, Scotland). Background counts from cover slips without cells were subtracted from all determinations in the final graphic presentation of the results.

*Fc Membrane Receptor Marker.* This was tested by the ingestion of IgG-coated sheep erythrocytes (SRBC). Rabbit anti-SRBC IgG was obtained from Cordis Laboratories, Miami, Fla. This particular batch was a generous gift from Dr. Z. Cohn's laboratory at The Rockefeller University, New York. SRBC were obtained from the Statens Institutt for Folkehelse, Oslo, Norway, and were kept in Alsever's solution and used within 1 wk. The SRBC were washed carefully and suspended at 5% in HEPES-buffered MEM (HMEM) before being coated with IgG for 15 min at 37°C. The IgG-SRBC were then washed and resuspended at 0.5%. 0.5 ml of this 0.5% suspension was added to each of the 35 mm petri dishes containing cultured K cells, rinsed, and given 1 ml HMEM. The macrophage-SRBC mixture was left at 37°C for 30 min, rinsed gently, given a hypotonic shock with distilled water for 30 sec to lyse attached but uningested erythrocytes, and then fixed for phase-contrast microscopy. The Fc receptor was tested on 1-day and 3-day cultures.

*Morphology.* The cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, with 0.1 M sucrose. After rinsing, cover slips were mounted in Aquamount (Edward Gurr Ltd., London,

England), and examined under phase contrast in a Zeiss Photo microscope (Carl Zeiss, Inc., Wuerttemberg, West Germany). For transmission electron microscopy 1 day monolayer cultures of K cells were fixed in 1% glutaraldehyde in phosphate buffer, pH 7.4 410 mosM and postfixed in phosphate-buffered 1% OsO<sub>4</sub>, pH 7.3 (300 mosM). They were dehydrated in alcohol, and the monolayers detached from the plastic petri dishes during the propylene oxide step, and are pelleted and embedded in Epon. The blocks were sectioned on an LKB ultratome III (LKB, Stockholm, Sweden), grids were stained with lead citrate, and examined in a Philips 201 (Philips, Eindhoven, The Netherlands) EM at a magnification of 6,000 times and 8,000 times. For scanning electron microscopy the cells were postfixed in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer, dehydrated in ethanol, and transferred to amyloacetate. The preparations were critical point dried with a Hitachi HCP-1 (Hitachi Ltd., Tokyo, Japan) and then coated with carbon and gold with a Hitachi HUS 4GB evaporation unit. The specimens were examined in a Hitachi HHS 2R scanning microscope at a primary magnification of 1,000 × 30,000 times.

## Results

*Recovery of Kupffer cells.* The initial cell suspension contained  $9.5 \times 10^8$ – $11.3 \times 10^8$  cells/rat (Table I). There were 5–10% dead cells as judged by trypan blue uptake. Differential counts gave  $660 \times 10^6$ – $770 \times 10^6$  parenchymal

TABLE I  
*Preparation of K Cells by the Pronase Method. Results of One Typical Experiment (170-g rat) Showing the Number of Parenchymal Cells (PC), NPC, and K Cells (KC) at Each Step of the Preparation Procedure*

	PC (10 <sup>6</sup> )	NPC (10 <sup>6</sup> )	KC (10 <sup>6</sup> )
Initial cell suspension	710	308	
After 1 h pronase treatment	0	328	
After centrifugation	0	183	
Cells attached to glass	0	41	41

cells and  $290 \times 10^6$ – $358 \times 10^6$  smaller NPC (18–24.5%). In experiments where carbon had been injected intravenously before liver perfusion under 10% of the initial cell suspension contained intracellular carbon (Fig. 1).

After pronase treatment, no viable parenchymal cells were seen. NPC are quantitatively recovered, ranging from  $360 \times 10^6$  to  $380 \times 10^6$ , and 40% of the total cells contained carbon in their cytoplasm. In some experiments the number of NPC seems to increase somewhat during pronase treatment. This is most likely due to removal of parenchymal cells which permits easier recognition of NPC. There was no loss of carbon-containing cells during pronase treatment. Repeated washing and resuspension of the pronase digest removed up to 40% of the NPC but none of the K cells (Fig. 2). This means that most of the cells lost during the washing procedure were the smaller endothelial cells and/or fibroblasts, and this step caused in effect an enrichment of K cells.

DNA assays in the cell suspension before plating ( $0.5 \times 10^6$ /ml/trough) gave 4–4.2  $\mu$ g/trough. After overnight culture and thorough rinsing to remove unattached cells 0.95  $\mu$ g DNA could be recovered from each culture dish. This corresponds to a number of  $1.18 \times 10^5$  adhering cells in each culture dish, or about 23% of the total cells seeded. A comparable figure for mouse peritoneal

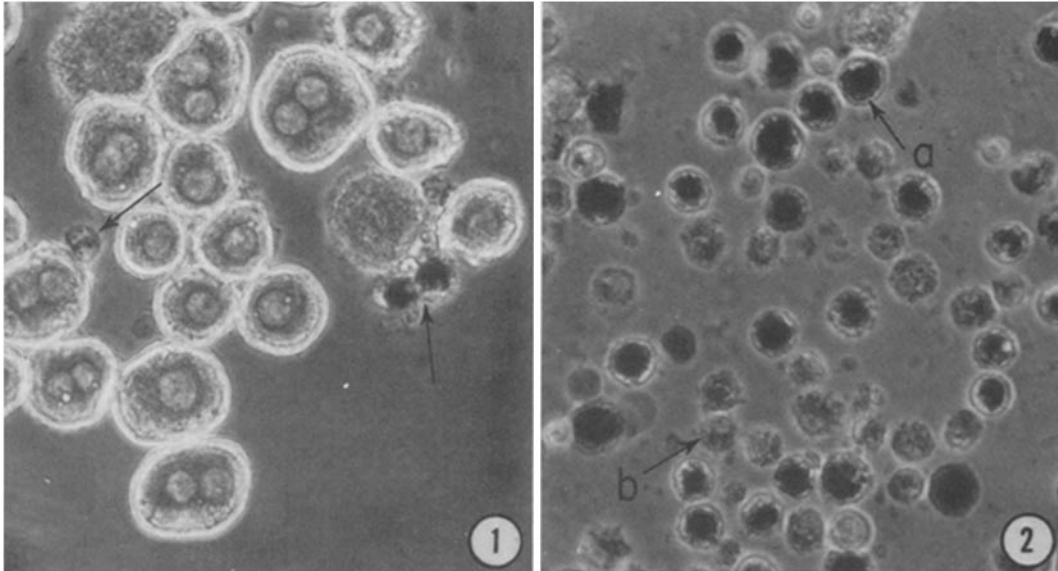


FIG. 1. The initial liver cell suspension from a rat preinjected with carbon. Light microscopy ( $\times 400$ ). The majority of cells are large and often polynucleated P cells. Nonparenchymal cells are much smaller and often in clusters or attached to debris (arrow). Some of the latter cells contain carbon in the cytoplasm.

FIG. 2. Final NPC suspension after pronase digestion of the initial liver cell suspension. Light microscopy ( $\times 400$ ). All P cells are selectively destroyed after 60–90 min of pronase treatment. The NPC are washed and concentrated. About 40% contain ingested carbon particles (arrow a) and are presumably K cells as contrasted to other NPC which do not ingest carbon (arrow b).

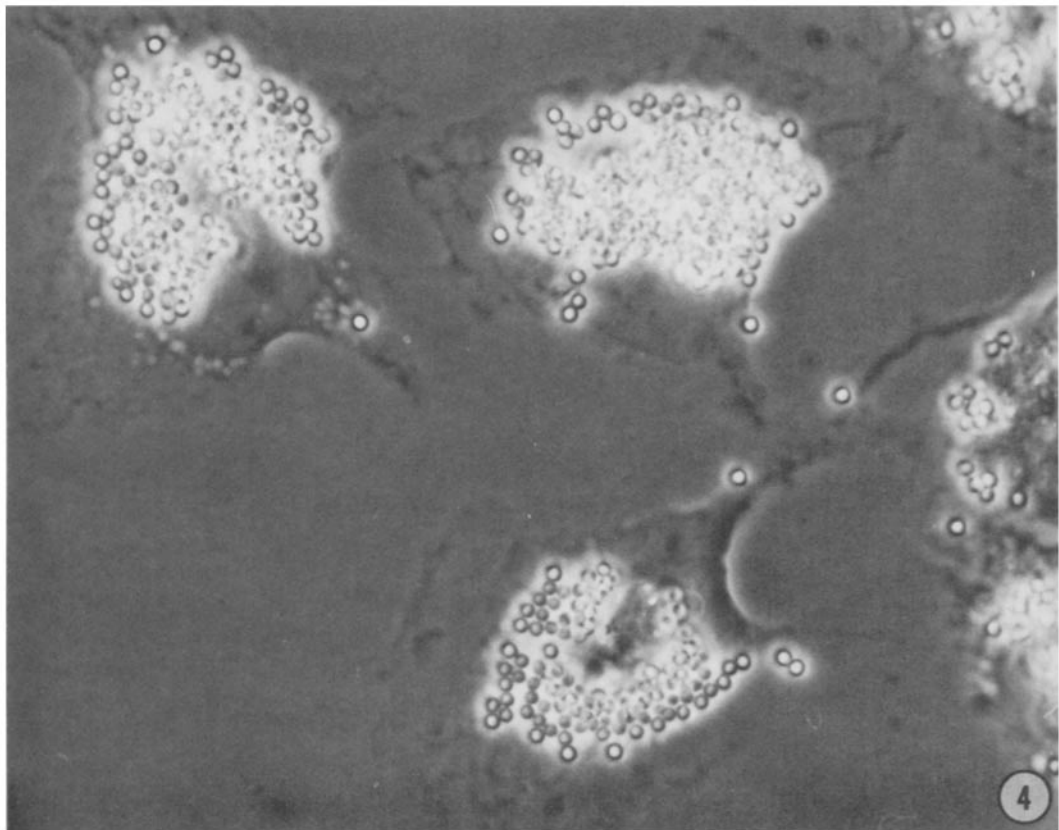
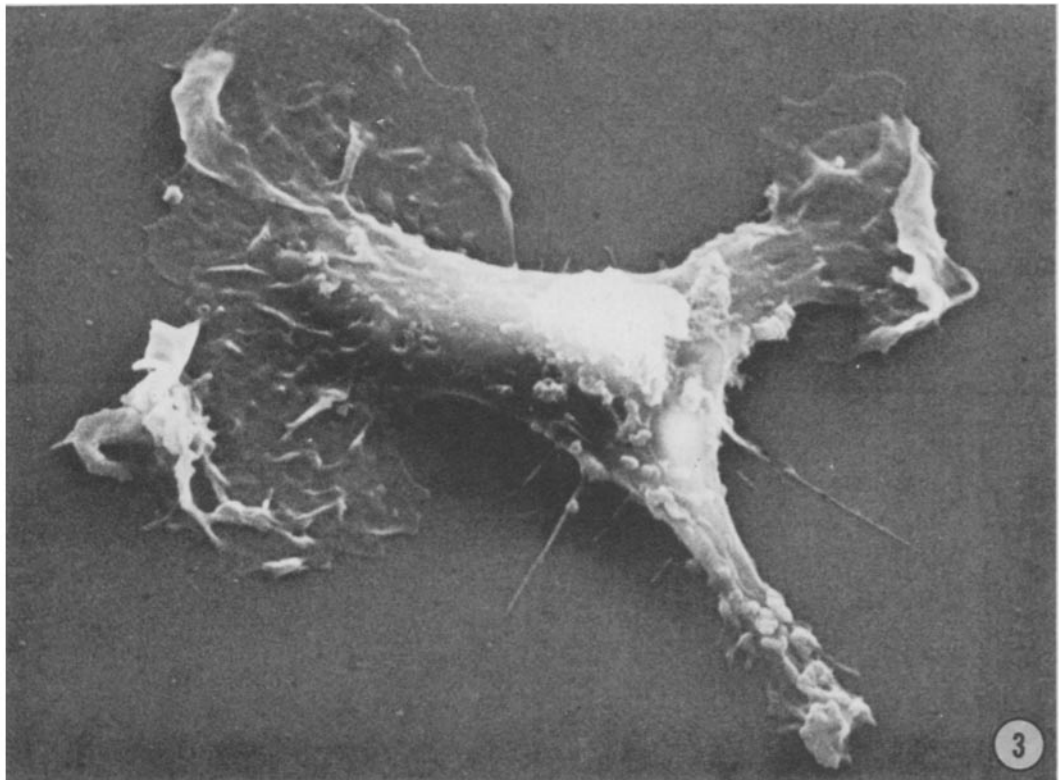
macrophages after 2 h in culture was 31% of the total cell suspension in peritoneal fluid.

*Morphology.* NBCS was found to be most consistent in giving good cultures of K cells. Rat serum was occasionally superior, and less serum was needed; but the problem of variability is undesirable. Unless otherwise stated, all K cells used in tests were cultured in 30% NBCS. The appearance of cultured K cells under the phase-contrast microscope was very similar to peritoneal macrophages. With the transmission electron microscope the cells displayed the usual features of cell ultrastructure.

The scanning electron microscope also revealed an appearance very similar to peritoneal macrophages (Fig. 3). The cells had a rounded or stellate configuration. There were a number of small projections on the surface, and ruffling was prominent along the edges of some of the cells.

FIG. 3. Scanning electron micrograph of a K cell after 24 h in vitro ( $\times 5,400$ ). The cell has an overall stellate outline with numerous thin projections and ruffling at the periphery.

FIG. 4. K-cell phagocytosis of latex particles. Phase-contrast microscopy ( $\times 1,000$ ). After a 3-h incubation with latex particles, over 99% of the cells in the monolayer contain strongly refractile, intracellular latex particles in the perinuclear region of the cytoplasm.



When the K cells were cultured for periods of 1 wk or more, there was a tendency in some cases for other cell types, originally present in low concentrations, to become more numerous and spread between the K cells. The phase-contrast morphology suggested that the majority of the contaminating cells were fibroblasts.

*Endocytosis Studies.* More than 99% of the cells could phagocytose latex particles (Fig. 4). Control cultures incubated at 4°C showed no intracellular latex particles. Studies with radioactive colloidal gold indicated that K cells are very actively pinocytosing. After 90-min incubation the cell-associated radioactivity was more than six times the amount in control cultures at 4°C (Fig. 5).

*Fc Receptor.* The results are summarized in Table II. The percentage of cells ingesting IgG-coated SRBC was about 80% for the first several days of culture. The increase in size of the K cells in culture is reflected in the fact that average particles ingested per cell increased from between 3 to 4 to over 10 on the 3rd day.

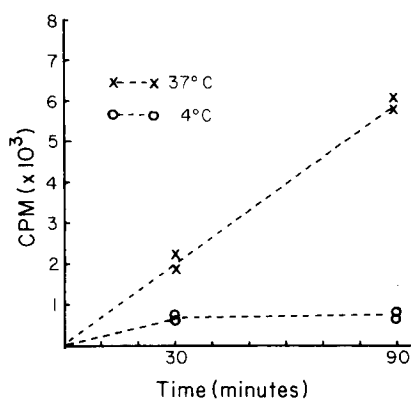


FIG. 5. Pinocytosis of colloidal  $^{198}\text{Au}$  by K cells in culture.

*Lysosomal Enzymes.* Table III gives the data on three lysosomal enzymes in the purified parenchymal cell suspensions, cell suspension recovered after pronase digestion (NPC), and in cells cultivated for 24 h in vitro (K cells). It can be seen that the K cell and the NPC have significantly higher specific activities than the parenchymal cells. However, there was little difference between K cells and total NPC.

## Discussion

If we accept the definition of K cells to be the mononuclear phagocytes of the liver, then the crucial test for both purity and yield of a K-cell preparation is its phagocytic ability. On this basis we claim to have up to 99% pure K-cell cultures. This figure is based on the finding with latex particles. With IgG-coated SRBC the figures run somewhat lower. It is not entirely clear what this means. It may mean that not all liver macrophages, K cells by definition, express the Fc receptor on their surfaces at any given time. Preliminary experiments with rat peritoneal macrophages indicate that also only a similar percentage of these cells

TABLE II  
*Effect of Rabbit AntiSRBC, IgG on the Phagocytosis of  
 SRBC by K Cells in Culture*

Test particles	Age of K cells (days in culture)	K cells ingesting test particles‡	Average number of particles ingested per K cell
		%	
Uncoated SRBC	1	2*	1*
IgG-coated SRBC	1	80	3.3
Uncoated SRBC	3	2*	1*
IgG-coated SRBC	3	84	12.6

‡ At least 100 K cells counted per slide.

\* Questionable inclusion in cytoplasm.

TABLE III  
*Protein and Specific Activities of Lysosomal Enzymes in K Cells (KC), Total NPC, and Parenchymal Cells (PC) from the Same Experiment as Table I. Specific Activities of Lysosomal Enzymes are Expressed in nmol/min/mg Protein*

	KC	NPC	PC
Protein ( $\mu\text{g}/10^6$ cells)	153.5*	109.3	1918.0
$\beta$ -glucuronidase	22.79	17.98	4.14
Acid DNAase	25.10	29.43	4.75
Cathepsin-D	9.00	7.05	0.94

\* Cell number based on DNA determination.

express the Fc receptor at a given time point. It may also mean that some of the cells capable of ingesting latex particles are other cell types devoid of Fc receptors and unrelated to the mononuclear phagocytic system. Thirdly, all Fc receptor-bearing cells may not have been recorded for technical reasons. Future studies will have to decide which of these possibilities is correct.

The yield is more difficult to assess since we do not know the number of K cells in the intact liver to start with. Weibel et al. (17) calculated on the basis of morphometrical data a total number of about  $3.02 \times 10^8$  NPC/100-g rat; and Jandl et al. (18), using chemical measurements of liver DNA arrived at a corresponding figure of  $3 \times 10^8/100$ -g rat. It is not entirely clear whether all authors refer to the same population of cells. We have obtained about  $2 \times 10^8$  NPC/100-g rat (Table I). This means that, if the figures from the literature are correct, we have been able to get two-thirds of the total liver NPC into suspension by the end of pronase digestion. Other reports have described up to about one-fourth of this yield ( $1.5 \times 10^8/300$ -g rat) (19). The cause of the high yield of NPC in our laboratory may be due to the fact that we performed the pronase

treatment on cell suspensions rather than on tissue pieces as carried out by some other researchers (19, 20).

Our data appear even better when we compare the final yield of K cells. The carbon experiments demonstrate that about 40% of our NPC after pronase treatment were K cells. This is in agreement with reports of biochemical identification of K cells in livers fixed by perfusion (6, 21, 22). In the experiment reported in Table I, this corresponded to about  $77 \times 10^6$  K cells/100-g rat. Other comparable figures from the literature range from  $2.11 \times 10^5$  cells/g liver corresponding to  $2 \times 10^6$  cells/100-g rat (23) to about  $12.5 \times 10^6$  cells/100-g rat (19).

Of the cells seeded into tissue culture dishes about 23% attached. Since the cell suspension used for seeding contained about 40% K cells, it follows that more than 50% of the K cells eventually attached to the cover slips. It may be possible to improve the figure by modifying the handling of the cells and by finding optimal *in vitro* conditions. This optimism is based on the assumption that all K cells are able to attach under ideal conditions. However, it may not be so: the results with mouse peritoneal macrophages subjected to a minimum of handling, indicate that during any given time period, only a fraction of even fully developed phagocytes can attach to the culture dish. Thus, in our hands about 30% of peritoneal cells attach to the dish whereas recent reports indicate that about 50% of the peritoneal cells are macrophages (24).

The scanning electron microscopy morphology and the efficiency of pinocytosis and phagocytosis stress the close similarity of K cells to other macrophages, and bring additional evidence that the two cell types are closely related (25). As a matter of fact the majority of K cells could not be distinguished from cultivated peritoneal macrophages on the basis of morphology and avidity of endocytosis. It remains for future studies to establish whether the finer details of their functioning will confirm this similarity.

The specific activities of the three lysosomal enzymes studied was significantly higher in NPC and K cells than in whole liver or purified parenchymal cells. It may be a little surprising that there was so little difference between K cells and NPC. This may indicate that the K cells do not have full degradative capacity in the intact liver of an animal kept under standard laboratory conditions, but that the capacity may develop upon challenge either *in vivo* or *in vitro*.

The importance of the liver macrophage in physiological functions such as detoxification and immune surveillance cannot be exaggerated. This system we have now developed can enable closer studies of the K cells regarding both biochemical analyses and functional abilities. The former can lead to elucidation of much of the inconsistency encountered hitherto by biochemists studying whole livers prepared in different ways. Studies on surface receptors, metabolic events in endocytosis, lysosomal contents, etc. will provide comparison with the accumulated data already available on the peritoneal macrophage.

## Summary

Collagenase perfusion of the liver followed by pronase treatment of the cell suspension thus obtained gave a quantitative recovery of viable nonparenchymal



liver cells (NPC). From these NPC, Kupffer (K) cells can be purified by attachment to tissue culture dishes. Tail vein injection of carbon 1-2 h before liver perfusion permitted stepwise calculation as well as visualization of carbon-containing K cells. When these K cells have been put into tissue culture medium with serum and incubated overnight, they exhibit typical macrophage characteristics.

Phase-contrast and transmission electron microscopy showed typical macrophage morphology and scanning electron microscopy revealed well-spread cells with cytoplasmic projections and ruffled membranes. Endocytosis studies using radioactive colloidal gold and inert latex particles also indicated that these cells are highly active in pinocytosis and phagocytosis. Further characterization of K cells is the identification of Fc receptor on their membranes. Studies on lysosomal enzymes showed that purified K cells possess higher specific activities in  $\beta$ -glucuronidase, acid DNase, and cathepsin D than in purified parenchymal cells.

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