QUANTITATIVE DETERMINATION OF IN VIVO ENDOCYTOSIS BY RAT LIVER KUPFFER AND ENDOTHELIAL CELLS FACILITATED BY AN IMPROVED CELL ISOLATION METHOD

D. P. PRAANING-VAN DALEN and D. L. KNOOK
Institute for Experimental Gerontology TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands

Received 29 March 1982

1. Introduction

The sinusoidal cells in the mammalian liver represent a major part of the reticuloendothelial system and are responsible for the clearance of various substances. The main types of sinusoidal cells are Kupffer, endothelial and fat-storing cells. In the rat liver, both Kupffer and endothelial cells play an important role in the endocytosis of injected ligands such as glycoproteins [1,2].

Procedures developed for the preparation of highly purified Kupffer and endothelial cells [3,4] offer the possibility to determine the amount of ligand endocytosed by each of the cell types prior to their isolation. However, during the liver perfusion and subsesequent incubation of the cells at 37°C, degradation of the intracellular ligands can occur. Furthermore, ligands released from damaged cells may be endocytosed anew during the isolation procedure.

Here, we present a method for the isolation of Kupffer and endothelial cells at low temperature. The endocytosis of colloidal albumin and ahexosaminoorosomucoid (AHOR), ligands known to be endocytosed by both Kupffer and endothelial cells [2,5], was quantified. The results obtained demonstrate that with the procedure described the amount of ligand recovered in the isolated sinusoidal cells is several times higher than with the 37°C procedure. Both Kupffer and endothelial cells exhibit a similar endocytic capacity for colloidal albumin, while endothelial cells are twice as active as Kupffer cells in the endocytosis of injected AHOR.

Abbreviations: AHOR, ahexosaminoorosomucoid; GBSS–BSA, Geys' balanced salt solution containing bovine serum albumin

2. Materials and methods

2.1. *Preparation of ligands*

Bovine serum albumin was labeled with ^{125}I as in [6]. The labeled albumin was heat-aggregated according to [7]. AHOR was kindly provided by Dr C. Steer (NIH, Bethesda, Maryland MD) and labelled with ^{125}I by the chloramine-T method [8].

2.2. *Injection and plasma clearance of the ligands*

Colloidal albumin (405 μg) or AHOR (24 μg) per 100 g body wt were injected intravenously (jugular vein) into 3-month-old BN/BiRij rats (160 g av. body wt). Under mild ether anesthesia, at least 5 blood samples were taken from the tail vein at evenly distributed time points after injection to determine the half lives and the clearance rates of the ligands from the blood. The chosen time interval between injection of ligands and sacrifice of the animal was 5–6 min to avoid degradation of the ligands in the cells before the start of the cell isolation procedure.

2.3. *Isolation and purification of Kupffer and endothelial cells*

The rat liver was perfused in situ (10 ml/min) with Geys' balanced salt solution (GBSS) at 37°C for 3 min and subsequently at 10°C for 10 min with GBSS containing 0.2% pronase E (Merck), followed by GBSS containing 1.3% bovine serum albumin (GBSS–BSA) at 10°C for 2 min. After excision of the liver, Glisson's capsule was carefully removed. The cells were brought into suspension by shaking the liver briefly in ice-cold GBSS–BSA and scraping the tissue lightly with a spatula. The pH was adjusted to 7.4 with 1 N NaOH. The cell suspension was filtered through nylon gauze and the filtrate was centrifuged at 300 × g for 10 min

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75 © 1982 Federation of European Biochemical Societies

at 4°C. The cells in the pellet were freed of erythrocytes by density centrifugation in GBSS containing 17.5% (w/v) metrizamide at 1400 × g for 15 min at 4°C [3]. After centrifugation, the sinusoidal cells, present in the top layer of the metrizamide solution were washed with GBSS–BSA at 300 × g for 10 min (4°C). The pellet was diluted in GBSS–BSA and used as a sinusoidal cell preparation for some of the experiments. Kupffer and endothelial cells were further purified by centrifugal elution in a Beckman JE-6 elution rotor at 4°C with GBSS–BSA as the elution fluid [3]. Four successive fractions were collected at a constant rotor speed of 2500 rev./min: lymphocyte, endothelial cell, intermediate cell and Kupffer cell fraction at flow rates of 13.5, 20.0, 23.5 and 40.0 ml/min, respectively. To compare the relative amount of colloidal albumin present in cells isolated by different methods, some experiments were performed using the pronase E isolation method in [3]. The cells were identified cytochemically [9] and by their electron microscopic morphology [9]. Cell counts were performed with a hemocytometer. Cell viability was estimated from the percentage of cells which excluded 0.25% trypan blue.

3. Results

3.1. *Characterization of the cell preparations*

The main difference between this isolation method and the pronase E method in [3,4] is the reduced temperature during the entire isolation procedure. At 10°C, pronase is apparently still able to digest the parenchymal cells, since the total sinusoidal cell suspension contained only 1–2% parenchymal cells before elution. The amount of sinusoidal cells recovered from a liver of ~5 g varied from 160–200 × 10^6 cells, of which ~20% were Kupffer cells, 27% lymphocytes and 53% endothelial cells. After elution, >90% of the cells could be recovered. The viability of the eluted cells was more than 90%. The average purity of the endothelial and Kupffer cell fractions was 90% and 80%, respectively.

3.2. *Plasma clearance and liver uptake of colloidal albumin and AHOR after intravenous injection*

On the basis of the amount of colloidal albumin or AHOR injected and an estimated plasma volume of 3.13 ml/100 g body wt of rat [10], original plasma concentrations of the ligands were calculated. Using

Table 1
Plasma clearance and liver uptake of colloidal albumin and ahexosaminoorosomucoid after intravenous injection

	Colloidal albumin (n = 3)	Ahexosamino-orosomucoid (n = 3)
Time interval	5.4 min	5.9 min
Half-life in plasma	110 s	108 s
Plasma concentration:		
average	42.6%	40.0%
at time of sacrifice	14.3%	11.0%
% of dose in liver	61.4%	56.9%

these values, disappearance curves of the ligands from the plasma were determined (not shown). Both ligands were cleared from the plasma at a high rate. Table 1 shows that the half-lives, given as the period of time in which 50% of the ligands disappeared from the plasma, were <2 min. An exact determination of the average concentration of the ligands during the time interval between injection and sacrifice of the animals was required for further calculations (see table 3). These values, calculated by means of the disappearance curves, are expressed in table 1 as percentages of the original plasma concentrations. For both ligands, the average concentration was ~40%. At the time of sacrifice at 5–6 min after injection (see table 1), 86% and 89% of the initial dose of colloidal albumin and AHOR were cleared from the plasma; 61.4% and 56.9% of the two injected ligands were present in the liver, which represent 72% and 64% of the dose cleared from the plasma at that time.

3.3. *Quantification of endocytosis by Kupffer and endothelial cells*

Attempts to determine the amount of in vivo endocytosed colloidal albumin in sinusoidal cells isolated by a 37°C pronase E method resulted in a considerable loss of radioactivity from the cells. About 90% of the amount of colloidal albumin present in the liver at the start of the perfusion was lost in a trichloroacetic acid-soluble form during isolation and separation of the cells. When chloroquine, an inhibitor of lysosomal degradation, was injected prior to the ligand (table 2, method I), only a minor reduction in the loss of degraded ligand was observed. Even when the injected dose of chloroquine was increased and chloroquine was also added to the isolation medium (table 2, method II), the loss of label was

Table 2
Recovery of cells and of colloidal albumin in sinusoidal cell fraction

Method	Yield of Kupffer and endothelial cells (10^6 g/liver)	Colloidal albumin	
		In cell fraction	Lost in trichloroacetic acid-soluble form during isolation
I (n = 2)	22.4	5.7	76.8
II (n = 2)	24.6	15.7	74.0
III (n = 3)	28.9	38.5	0.9

Sinusoidal cells were isolated as in [3] (methods I and II) or in section 2 (method III): method I, 0.1 ml 10 mM chloroquine was injected 10 min prior to the ligand; method II, 0.1 ml 100 mM chloroquine was injected and 1 mM chloroquine was present in the isolation medium. Values for the amounts of colloidal albumin are given as percentages of the total amount present in the liver at the start of the isolation procedure

still 74%. A higher concentration of chloroquine could not be used, since this would have a damaging effect on the cells [11]. However, when cells were isolated by the low temperature method (table 2, method III), <1% of the total amount of ligand was lost due to degradation.

With the low temperature method, the recovery of ligand in the sinusoidal cell fraction was increased from 5.7–38.5%, while the recovery of Kupffer and endothelial cells in the sinusoidal cell prearation did not differ significantly (table 2) and represented ~50% of the cells present in the intact liver [3]. From the results obtained with the sinusoidal cells isolated according to the low temperature method, it follows that ~80% of the colloidal albumin in the liver was present in the Kupffer and endothelial cells. The remainder might have been present in the parenchymal cells [5].

The new isolation method was applied for the quantification of in vivo endocytosis per sinusoidal cell class. Kupffer and endothelial cells were purified by centrifuged elution. Table 3 shows the amount of in vivo endocytosed colloidal albumin and AHOR by both cell types expressed per 10^6 cells. The capacity of the endothelial cells to endocytose colloidal albumin is the same as that of the Kupffer cells, whereas the amount of AHOR endocytosed by endothelial cells is twice as high as found in Kupffer cells. A direct comparison of the endocytosis of the 2 ligands is possible only when the injected dose and the avarage plasma concentration are taken into account. Therefore, table 3 also compares the rate of endocytosis,

Table 3
Localization of colloidal albumin and ahexosaminoorosomucoid in purified Kupffer and endothelial cells

Ligands	Endothelial cells (A)		Kupffer cells (B)		Ratio (A/B)
	Amount endocytosed	Endocytic index	Amount endocytosed	Endocytic index	
Colloidal albumin	738	3740	683	3460	1.08
Ahexosamino-orosomucoid	34.6	2750	17.8	1420	1.94

The amount of endocytosed ligand is expressed in ng/10^6 cells. The endocytic index, expressed in μl plasma . 10^6 cells^{-1} . day^{-1}, was calculated on basis of incubation time, average plasma concentration and amount of ligand present per 10^6 endothelial or Kupffer cells. Values were corrected for the contribution of cross-contamination in the cell preparations (n = 3)

expressed by the endocytic index, in μl ligand containing liquid (i.e., the plasma) internalized by a unit quantity of cells (10^6) in a unit time (per day). The endocytic indices show that AHOR is less actively endocytosed than colloidal albumin by both cell types, but especially by the Kupffer cells.

4. Discussion

We present here a method for isolating rat liver Kupffer and endothelial cells at low temperature and which prevents degradation of in vivo endocytosed ligands. This method makes possible the quantitative determination of in vivo endocytosis, in contrast to 37°C methods in which degradation of ligands is prevented by preloading the cells with inhibitors of lysosomal degradation, such as chloroquine. It appeared that complete prevention of the degradation could not be accomplished in this way. Furthermore, such inhibitors can reduce the viability of the cells [11] and might affect their endocytic capacity [12]. Another advantage of using a low temperature during the isolation procedure is the inhibition of endocytosis [13], which makes intercellular redistribution of ligands very unlikely.

We employed our isolation method to determine the in vivo endocytosis of two selected ligands, colloidal albumin and AHOR. Experiments with other substances such as acetylated human low density lipoprotein (J. F. Nagelkerke, K. P. Barto and Th. J. C. van Berkel, in preparation) and cytoplasmic or mitochondrial malate dehydrogenase (M. K. Bijsterbosch, A. M. Duursma, J. M. W. Bouma and M. Gruber, in preparation) indicate that these ligands can also be quantitatively recovered in the liver cells isolated at low temperature. Moreover, the low temperature isolation method is also suitable for in vitro studies on receptor-mediated endocytosis, since cells so isolated still possess intact receptors for several ligands such as colloidal albumin and AHOR (unpublished).

Our results show that intravenously injected colloidal albumin and AHOR could be detected not only in Kupffer but also in endothelial cells after their isolation. Although endothelial cells are smaller than Kupffer cells [3], they prove to have the same endocytic capacity for colloidal albumin and twice as high for AHOR as Kupffer cells. These results could be confirmed in studies on the in vitro endocytosis of the two ligands by Kupffer and endothelial cells isolated by the new method (unpublished). Since there are ~3-times more endothelial than Kupffer cells present in the liver [3], the relative contribution of the endothelial cells to the endocytosis of colloidal albumin and AHOR by the liver is 3- and 6-times higher, respectively. Kupffer cells are often believed to be responsible for endocytosis of various ligands. Extending the results in [2,5], this study shows that endothelial cells in general can make an important contribution to endocytosis and catabolism of macromolecules.

Acknowledgements

These investigations were supported in part by the Foundation for Medical Research FUNGO which is subsidized by the Netherlands Organization for Advancement of Pure Research (ZWO). The authors are indebted to Ms Christa de Ruiter for skillful technical assistance and thank Dr Th. J. C. van Berkel for critically reading the manuscript.

References

[1] Schlesinger, P. H., Doebber, T. W., Mandell, B. F., White, R., De Schryver, C., Rodman, J. S., Miller, M. J. and Stahl. P. (1978) Biochem. J. 176, 103–109.
[2] Hubbard, A. L., Wilson, G., Ashwell, G. and Stukenbrok, H. (1979) J. Cell Biol. 83, 47–64.
[3] Knook, D. L. and Sleyster, E. Ch. (1976) Exp. Cell Res. 99, 444–449.
[4] Knook, D. L., Blansjaar, N. and Sleyster, E. Ch. (1977) Exp. Cell Res. 109, 317–329.
[5] Praaning-van Dalen, D. P., Brouwer, A. and Knook, D. L. (1981) Gastroenterology 81, 1036–1044.
[6] Helmkamp, R. W., Goodland, R. L., Bale, W. F., Spar, J. L. and Mutschler, L. E. (1960) Cancer Res. 20, 1495–1500.
[7] Benacerraf, B., Biozzi, G., Halpern, B. N., Stiffel, C. and Mouton, D. (1957) Brit. J. Exp. Pathol. 38, 35–48.
[8] Greenwood, F. C., Hunter, W. M. and Glover, J. S. (1963) Biochem. J. 89, 114–123.
[9] Knook, D. L. and Sleyster, E. Ch. (1977) in: Kupffer Cells and Other Liver Sinusoidal Cells (Wisse, E. and Knook, D. L. eds) pp. 273–288, Elsevier Biomedical, Amsterdam, New York.
[10] Munniksma, J., Noteborn, M., Kooistra, T., Stienstra, S., Bouma, J. M. W., Gruber, M., Brouwer, A., Praaning-van Dalen, D. and Knook, D. L. (1980) Biochem. J. 192, 613–621.
[11] Van Berkel, T. J. C., Nagelkerke, J. F. and Kruyt, J. K. (1981) FEBS Lett. 132, 61–66.
[12] Sando, G. N., Titus-Dillon, P., Hall, C. W. and Neufeld, E. (1979) Exp. Cell Res. 119, 359–364.
[13] Weigel, P. H. and Oka, J. A. (1980) J. Biol. Chem. 256, 2615–2617.