INFECTION AND IMMUNITY, Aug. 1981, p. 620-622
0019-9567/81/080620-03$02.00/0

Vol. 33, No. 2

Lysosomal Enzyme Content of Kupffer and Endothelial Liver Cells Isolated from Germfree and Clean Conventional Rats

DICK L. KNOOK,* CHRIS BARKWAY, AND ELISABETH C. SLEYSTER

Institute for Experimental Gerontology TNO, 2280 HV Rijswijk, The Netherlands

Received 23 January 1981/Accepted 21 April 1981

Rats kept under germfree conditions showed lower specific lysosomal enzyme activities in liver endothelial cells, but not in Kupffer cells.

The Kupffer and endothelial cells in the sinusoidal areas of the liver may play a central role in the animal's defense against circulating microorganisms and other pathogenic agents (20). The phagocytosis of bacteria and the bactericidal activity of Kupffer cells have already been clearly demonstrated (5-7, 13). Kupffer cells are also almost exclusively responsible for the clearance of bacterial products such as endotoxin (D. P. Praaning-van Dalen, A. Brouwer, and D. L. Knook, submitted for publication). Although the endothelial cells do not show phagocytic activity, it has recently become evident that they are able to clear several test substances used to measure the activity of the hepatic reticuloendothelial system (Praaning-van Dalen et al., submitted for publication). In addition, endothelial cells show a preferential uptake of circulating glycoproteins (8) and mucopolysaccharides (see reference 12).

The material endocytosed by Kupffer and endothelial cells is exposed to their well-developed lysosomal digestion apparatus (12). In view of the role of both Kupffer cells and endothelial cells in the clearance function of the reticuloendothelial system, it was of interest to establish whether the germfree condition of rats was associated with alterations in the lysosomal enzyme activities of these cells. To accomplish this, highly purified suspensions of Kupffer and endothelial cells were prepared from germfree and clean conventional rats, and a number of lysosomal enzymes, selected for their role in the hydrolysis of a variety of substrates, were assayed for in the purified cell preparations.

Female BN/BiRij rats, 3 months of age, were obtained from the germfree colony of the REP-Institutes in Rijswijk or from our clean conventional aging colony. In the latter case, the rats were raised under specific-pathogen-free conditions (14). The bacteriological background of these rats consisted of "a colonization-resistant flora" (18, 19) and nonpathogenic strains of *Enterobacter cloacae*, *Staphylococcus albus*, and *Streptococcus faecalis*. After the age of about 6 weeks, these young rats were maintained under clean conventional conditions (14) until the age of 3 months, when they were used for the experiments. The germfree rats had somewhat lower body and liver weights than the clean conventional rats, but the ratio of liver weight versus body weight was unaltered (Table 1). Sinusoidal cells were isolated by pronase perfusion and incubation of the liver (10). Erythrocytes and cell debris were removed by centrifugation in a metrizamide solution with a density of 1.089 g/cm^3 at 21°C (10). Kupffer and endothelial cells were separated by centrifugal elutriation (10, 12). Based on cytochemical and ultrastructural characteristics (11), the purity of the endothelial cell fraction was at least 90%, whereas that of the Kupffer cell fraction ranged from 70 to 83% depending on the experiment. In all cases, the viability of the cells as based on the exclusion of 0.25% trypan blue was at least 93%. Kupffer cells from germfree rats had a slightly lower protein content in comparison with those prepared from clean conventional rats. The yield of Kupffer and endothelial cells from conventional and germfree animals was comparable (Table 1). The freshly purified cell suspensions were incubated with 0.05% Triton X-100 for 20 min at 0°C, and the activities of the following lysosomal enzymes were determined: acid phosphatase (EC 3.1.3.2), cathepsin D (EC 3.4.23.5), aminopeptidase B (EC 3.4.12A.1), *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), and arylsulphatase B (EC 3.1.6.1); the procedures were as previously reported (12).

The lysosomal enzyme activities of endothelial and Kupffer cells, expressed per milligram of cellular protein, are given in Table 2. Endothelial cells from germfree rats exhibited lower specific enzyme activities than those obtained from clean conventional animals. Similar differences were observed when the enzyme activities were expressed per cell, since endothelial cells from germfree and clean conventional rats had the same protein content (Table 1). Kupffer cells from germfree and clean conventional rats showed no significant differences in the specific

TABLE 1. *Characteristics of endothelial and Kupffer cells from clean conventional and germfree BN/BiRij rats*[a]

Rat	Rat body wt (g)	Liver wt (g)	Cell yield ($\times 10^6$) per g of liver		Protein content (μg) per 10^6 cells	
			Endothelial cells[b]	Kupffer cells[b]	Endothelial cells[b]	Kupffer cells[b]
Clean conventional	153 ± 1	5.0 ± 0.3	14.19 ± 1.21	6.95 ± 0.60	46 ± 3	111 ± 5
Germfree	141 ± 3	4.6 ± 0.4	14.93 ± 1.24	6.22 ± 1.21	45 ± 6	86 ± 11

[a] Average ± standard error from at least four separate experimental determinations.
[b] Isolated cells purified by centrifugal elutriation.

TABLE 2. *Specific lysosomal enzyme activities in isolated endothelial and Kupffer cells from BN/BiRij rats kept under different environmental conditions*

Enzyme investigated	Enzyme activity[a] in:			
	Endothelial cells		Kupffer cells	
	Clean conventional	Germfree	Clean conventional	Germfree
Acid phosphatase	165.3 ± 21.6	60.9 ± 25.0[b]	115.3 ± 14.6	113.3 ± 44.1
Cathepsin D	6.4 ± 0.4	3.9 ± 0.7[b]	17.0 ± 2.6	21.2 ± 2.6
Aminopeptidase B	9.2 ± 1.2	6.4 ± 0.4	11.3 ± 1.7	9.4 ± 0.4
N-Acetyl-β-D-glucosaminidase	299.0 ± 33.0	127.7 ± 21.9[b]	151.6 ± 8.3	144.7 ± 28.6
Arylsulphatase B	149.4 ± 18.0	99.7 ± 16.1	48.1 ± 10.4	52.8 ± 12.8

[a] Enzyme activities are expressed as nanomoles of 4-methylumbelliferone (acid phosphatase, *N*-acetyl-β-D-glucosaminidase), nanomoles of tryptophan (cathepsin D), nanomoles of β-naphthylamine (aminopeptidase B) or nanomoles of nitrocatechol (arylsulphatase B) released per minute per milligram of protein at 37°C. Data are mean ± standard error of four different cell preparations.
[b] $P < 0.01$ versus clean conventional.

activities of the lysosomal enzymes investigated (Table 2). Electron micrographs of Kupffer cells from germfree rats showed an abundance of lysosomal structures which varied in both size and structure. Similar abundance and heterogeneity of lysosomal structures have been observed for Kupffer cells prepared from clean conventional rats (10).

There is only one previous report in which liver lysosomal enzymes of germfree and conventional rats were compared (2). This showed that the levels of the lysosomal enzymes β-glucuronidase, arylsulphatase A, and acid deoxyribonuclease in nonparenchymal cell homogenates from germfree rats were lower than those in conventional rats. Those results are made confusing, however, by the use of the terms "nonparenchymal cells" and "Kupffer cells" interchangeably as well as by the use of an isolation technique which has proved to yield a nonparenchymal cell preparation with a low percentage of Kupffer cells (11). Therefore, the reported differences in activities of lysosomal enzymes determined in nonpurified nonparenchymal cells from germfree and conventional rats can be explained on the basis of our data in Table 2 to be due to lower enzyme activities in endothelial cells and not in Kupffer cells from germfree rats.

The question of why lysosomal enzyme activities are not changed in Kupffer cells from germfree rats may be answered as follows. Some Kupffer cell functions studied in germfree mice have found to be unaltered as compared with those in conventional animals. The capacity to clear nonviable ^{32}P-labeled *Staphylococcus aureus* and *Escherichia coli* was not significantly changed in germfree mice (17). Also carbon uptake, which, depending on the size of the particles, is mainly a Kupffer cell function, was not decreased in germfree mice (17). Thus, the functional capacity of Kupffer cells to phagocytose seems not to be altered under germfree conditions. This is a reasonable assumption, since Kupffer cells play an important role in erythrophagocytosis and iron metabolism (20), processes which occur largely independent of the environment. Furthermore, even under germfree conditions, endotoxins are present in the sterilized food and will be removed from the venous blood by the Kupffer cells, which nearly exclusively take up endotoxin (Praaning-van Dalen et al., submitted for publication). The active role of Kupffer cells in all of these functions in germfree animals might explain the unchanged lysosomal levels in these cells.

In contrast to the findings for Kupffer cells,

lysosomal enzyme activities are lower in endothelial cells prepared from the livers of germfree rats. Endothelial cells are normally involved in the selective clearance of circulating glycoproteins, lipoproteins, and mucopolysaccharides (see reference 12). The lysosomal enzymes involved in the degradation of these macromolecules include the proteolytic enzyme cathepsin D and the glycosidase *N*-acetyl-β-D-glucosaminidase. Both enzymes are strongly reduced in endothelial cells in germfree rats. A less pronounced decline in activity was observed for aminopeptidase B. Unlike cathepsin D, which is primarily involved in the degradation of foreign proteins, aminopeptidase B may have a more general proteolytic function (9).

Why the lysosomal enzyme activities are decreased in endothelial cells is not yet clear. Little is known of the mechanism of the regulation of steady-state levels of lysosomal enzymes, but there are indications for an influence of several factors, including the quality and quantity of the endocytosed substrates and the contribution of endocytosed lysosomal enzymes originally present in the circulation. With respect to properties of the substrates, it can be mentioned that endothelial cells are most probably involved in the clearance of soluble antibody-antigen complexes from the circulation by means of their mannose specific receptor (3). Since germfree animals have a reduced number of immune complexes in their plasma (1), a reduced uptake of these complexes may lead to lower lysosomal enzyme levels in their endothelial cells.

A comparable situation can be expected for the possible contribution of endocytosed lysosomal enzymes. Several lysosomal glycosidases are specifically cleared from the circulation by endothelial cells via a mannose receptor (8, 15, 16). Since lysosomal glycosidases can be released by Kupffer cells during phagocytosis of, e.g., bacteria (20), the levels of circulating glycosidases in germfree rats may be lower due to the absence of infections. A decreased capacity to release lysosomal enzymes has also been observed for leukocytes of germfree animals (4). Therefore, a decreased clearance of lysosomal enzymes from the circulation may contribute to the observed lower levels of lysosomal enzymes in endothelial cells from germfree rats.

We thank Ingrid Boonstra for skilled technical assistance and Margreet de Leeuw for preparing the electron micrographs.

LITERATURE CITED

1. **Anderson, R. E., J. Scaletti, and J. L. Howarth.** 1972. Radiation-induced life shortening in germfree mice. Exp. Gerontol. **7**:289–301.
2. **Berg, T., and T. Midtvedt.** 1976. The influence of infection on the content of lysosomal enzymes in rat Kupffer cells and hepatocytes. Acta Pathol. Microbiol. Scand. Sect. A **84**:415–420.
3. **Day, J. F., R. W. Thornburg, S. R. Thorpe, and J. W. Baynes.** 1980. Carbohydrate-mediated clearance of antibody-antigen complexes from the circulation. The role of high mannose oligosaccharides in the hepatic uptake of IgM antigen complexes. J. Biol. Chem. **225**:2360–2365.
4. **Ferenčík, M., L. Bergendi, L. Mandel, F. Kovářů, J. Štefanovič, and Z. Ďuračková.** 1979. The lysosomal enzyme and antibacterial factors in leukocytes, serum and spleens of conventional and germfree piglets. Folia Microbiol. (Prague) **24**:701A.
5. **Friedman, R. L., and R. J. Moon.** 1977. Hepatic clearance of *Salmonella typhimurium* in silica-treated mice. Infect. Immun. **16**:1005–1012.
6. **Friedman, R. L., and R. J. Moon.** 1980. Role of Kupffer cells, complement, and specific antibody in the bactericidal activities in perfused livers. Infect. Immun. **29**: 152–157.
7. **Hauser, R., and B. E. Matter.** 1977. Localization of *E. coli* K-12 in livers of mice used for an intrasanguineous host-mediated assay. Mutat. Res. **46**:45–48.
8. **Hubbard, A., G. Wilson, G. Ashwell, and M. Stukenbrok.** 1979. An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. 1. Distribution of ^{125}I-ligands among the liver cell types. J. Cell Biol. **83**:47–64.
9. **Knook, D. L.** 1977. The role of lysosomal enzymes in protein degradation in different types of rat liver cells. Acta Biol. Med. Ger. **36**:1747–1752.
10. **Knook, D. L., N. Blansjaar, and E. C. Sleyster.** 1977. Isolation and characterization of Kupffer and endothelial cells from the rat liver. Exp. Cell Res. **109**:317–329.
11. **Knook, D. L., and E. C. Sleyster.** 1977. Preparation and characterization of Kupffer cells from rat and mouse liver, p. 273–288. *In* E. Wisse and D. L. Knook (ed.), Kupffer cells and other liver sinusoidal cells. Elsevier/North-Holland Biomedical Press, Amsterdam.
12. **Knook, D. L., and E. C. Sleyster.** 1980. Isolated parenchymal, Kupffer and endothelial rat liver cells characterized by their lysosomal enzyme content. Biochem. Biophys. Res. Commun. **96**:250–257.
13. **Mohelska, H., R. Molusa, M. Kubin, and K. Smetana.** 1975. Endocellular parasitism of *Mycobacterium avium* in rabbit liver. Exp. Pathol. **10**:122–131.
14. **Solleveld, H. A.** 1978. Types and quality of animals in cancer research. Acta Zool. Pathol. Antverp. **72**:5–18.
15. **Steer, C. J.** 1980. Carbohydrate recognition systems of sinusoidal liver cells: an update. Kupffer Cell Bull. **3**: 15–22.
16. **Steer, C. J., J. W. Kusiak, R. O. Brady, and E. A. Jones.** 1979. Selective hepatic uptake of human β-hexosaminidase A by a specific glycoprotein recognition system on sinusoidal cells. Proc. Natl. Acad. Sci. U.S.A. **76**:2774–2778.
17. **Thorbecke, G. J., and B. Benacerraf.** 1959. Some histological and functional aspects of lymphoid tissue in germfree animals. II. Studies on phagocytosis *in vivo*. Ann. N.Y. Acad. Sci. **78**:247–253.
18. **Van der Waaij, D., J. M. Berghuis-de Vries, and J. E. C. Lekkerkerk-van der Wees.** 1971. Colonization resistance of the digestive tract in conventional and antibody-treated mice. J. Hyg. **69**:405–411.
19. **Wensinck, F., and J. G. H. Ruseler-van Embden.** 1971. The intestinal flora of colonization-resistant mice. J. Hyg. **69**:413–421.
20. **Wisse, E., and D. L. Knook.** 1979. The investigation of sinusoidal cells: a new approach to the study of liver function. Prog. Liver Dis. **6**:153–171.