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IDENTIFICATION OF A RECEPTOR FOR SENESCENT ERYTHROCYTES ON LIVER MACROPHAGES

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SUMMARY: Isolated rat liver macrophages adherent to culture dishes phagocytize selectively senescent rat erythrocytes as well as in vitro aged rat erythrocytes but not young or freshly isolated erythrocytes. Since in vitro aged erythrocytes lack cell bound antibodies and phagocytosis occurs in serum free medium, antibodies do not appear to play a role. Phagocytosis as well as binding of old erythrocytes to liver macrophages is inhibited by N-acetyl-D-galactosamine and related monosaccharides (1 - 25 mM range) and by corresponding synthetic glycoproteins (10^{-6} - 10^{-10} M range) but not by D-mannose or N-acetyl-D-glucosamine. We conclude that recognition and phagocytosis of senescent and in vitro aged erythrocytes is mediated by the galactose particle receptor on rat liver macrophages.

Sequestration of senescent red blood cells (RBC) is thought to primarily take place in the liver, spleen and bone marrow (1 - 3). In the liver resident macrophages (Kupffer cells) have been shown in vivo to take up old erythrocytes (4). It has been suggested, on the basis of studies with monocytes, that macrophages are not able to recognize senescent RBC per se but need the attachment of specific autoantibodies to senescence-associated antigens on erythrocytes (5). In contrast to findings with monocytes we now present evidence that macrophages of the liver can directly bind and phagocytize senescent or in vitro aged RBC, without prior binding of erythrocyte autoantibodies. The receptor for old RBC on liver macrophages has been identified as a N-acetyl-D-galactosamine/D-galactose-specific lectin, the galactose-particle receptor.

ABBREVIATIONS: (BSA) bovine serum albumin, (EGTA) ethyleneglycol-bis-(ß-amino-ethylether)-N,N'-tetraacetic acid, (D-Fuc) D-fucose, (L-Fuc) L-fucose, (Gal) D-galactose, (GalNAc) N-acetyl-D-galactosamine, (Glc) D-glucose, (GlcNAc) N-acetyl-D-glucosamine, (Man) D-mannose, (RBC) erythrocytes.

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MATERIALS AND METHODS

Senescent erythrocytes were separated from young erythrocytes according to their differences in density by a method described by Murphy (6). In detail, 12 - 15 ml blood was collected from Wistar rats (200 - 250 g body weight) with a syringe containing heparin. After centrifugation at 1000 g and 4°C for 20 min the serum was collected and the buffy coat removed. The erythrocytes were resuspended in the serum and the centrifugation step was repeated. The final erythrocyte pellet, at least 5 ml, was centrifuged in round bottom tubes with a volume of 10 ml for 60 min at 30°C at 30 000 g in a fixed angle rotor. About 150 µl from top and bottom of the erythrocyte pellet was collected and diluted in modified Eagle's medium containing 10 mM sodium phosphate, pH 7.4, to a final concentration of 2×10^8 cells/ml. Erythrocytes aged in vitro were prepared by storing the cells for 2 weeks at 4°C as described by Kay (5). Control erythrocytes were prepared as follows. Freshly drawn rat blood was mixed with sodium citrate to prevent clotting. Erythrocytes were washed three times with modified Eagle's medium plus 10 mM sodium phosphate and adjusted to 1×10^8 cells/ml.

Liver macrophages were prepared from male Wistar rats weighing 150 - 180 g by liver perfusion with 0.6 % collagenase (Boehringer, Mannheim, FRG) as described previously (7). Macrophages were enriched by differential centrifugation. The final macrophage suspension contained about 5 % hepatocytes and 5 to 15 % endothelial cells. Viability of the liver macrophages was more than 98 % as determined by trypan blue exclusion. Density was adjusted to 2×10^6 liver macrophages per ml modified Eagle's medium.

Phagocytosis assay. Liver macrophages were allowed to adhere to tissue culture dishes (24 well clusters from Costar, Cambridge, USA) in serum-free RMPI 1640 for 20 min at 37°C with 5 % CO_2. Nonadherent cells were removed by gentle washing. Erythrocytes were added at a ratio of about 50 cells per liver macrophage. Cells were cocultured for 20 min at 37°C. In inhibition experiments inhibitors were added to Kupffer cells 1 min prior to addition of erythrocytes. Noninternalized erythrocytes were removed by lysis with distilled water and liver macrophages were fixed with 1.5 % glutaraldehyde. Phagocytosis was examined by light microscopy. The percentage of macrophages with 3 or more erythrocytes internalized was determined ("% phagocytosis"). Each determination was performed in duplicate with 150 to 200 macrophages per sample.

Binding assay. 50 µl of liver macrophages was mixed with an equal volume of erythrocytes in round bottom tubes. Cells were centrifuged at 80 g for 5 min at 6°C and incubated for 60 min at 10°C. Resuspension was performed by gentle shaking. Cells were stained with crystal violet and examined in a haemocytometer by light microscopy. The percentage of macrophages with three or more erythrocytes bound was determined. Inhibition studies were performed by preincubating liver macrophages with the inhibitor for 5 min on ice. Mono- and oligosaccharides were from Serva (Heidelberg, FRG) and from Sigma (München, FRG). Asialofetuin was a gift from Prof. Bauer (Berlin, FRG). Synthetic glycoproteins, i.e. bovine serum albumin (BSA) derivatized with monosaccharides were a generous gift from Dr. Y.C. Lee (Baltimore, USA). The synthetic glycoproteins were derivates of the amidino-type and contained the following amounts of monosaccharides per mole BSA: 44 mole of D-galactose (Gal-BSA), 37 mole of N-acetyl-D-galactosamine (GalNAc-BSA), 37 mole of D-glucose (Glc-BSA), 44 mole of N-acetyl-D-glucosamine (GlcNAc-BSA), 28 mole of L-fucose (L-Fuc-BSA) and 38 mole of D-mannose (Man-BSA). Defatted BSA was from Sigma (München, FRG).

RESULTS AND DISCUSSION

Our studies were performed in parallel with senescent erythrocytes and with in vitro aged erythrocytes. Both cell types have been shown to express the same senescent antigen(s) (8). A major difference between the two RBC

types, however, is, that on senescent erythrocytes the age-specific antigen(s) are partially complexed with autoantibodies whereas RBC aged in vitro under serum-free conditions are devoid of such antibodies. Kay has reported that monocytes phagocytize senescent RBC but not in vitro aged RBC unless the latter have been allowed to react with autoantibodies, present in normal sera (5). On repeating these experiments with liver macrophages we observe here, however, that these macrophages phagocytize senescent RBC as well as in vitro aged RBC in the absence of serum antibodies (Fig. 1). Phagocytosis of young or freshly drawn erythrocytes is negligible. These results indicate that liver macrophages, in contrast to monocytes, have the ability to directly recognize senescence-associated antigens on erythrocytes, i.e. erythrophagocytosis by Kupffer cells does not depend on prior reaction of opsonin with age-specific antigens.

In a second series of experiments we tried to define the macrophage receptor involved and the nature of the senescence-associated antigen(s). Since it has been often speculated that erythrocytes during aging undergo changes in the carbohydrate portion of membrane glycoconjugates (7, 9-13) we

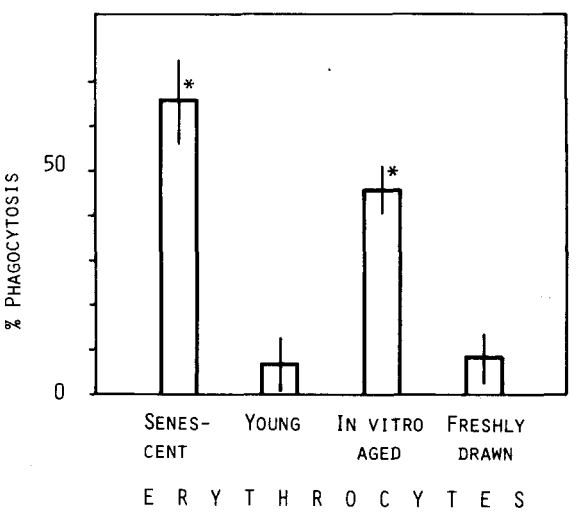

Fig.1 Phagocytosis of senescent and in vitro aged erythrocytes by liver macrophages in the absence of serum. Given is the percentage of liver macrophages which have internalized three or more erythrocytes per cell. Data are the average (+ standard deviation) from 4 experiments. The asterisks indicate significant difference at the $p < 0.001$ level (Student's t-test) to phagocytosis of young or freshly drawn erythrocytes.

have studied whether liver macrophages might recognize saccharide moieties of in vivo or in vitro aged erythrocytes. The results in Table 1 show that the addition of N-acetyl-D-galactosamine or D-galactose to the erythrophagocytosis assay specifically inhibits uptake of in vitro aged or senescent RBC. In contrast Fc-receptor mediated uptake of IgG-coated RBC is not inhibited under the same conditions (to be published).

Since it might be possible that these high carbohydrate concentrations (25 mM) have an effect on macrophage metabolism we have studied the process of senescent cell recognition separate from endocytosis in a cell adhesion assay at 10°C. About 50 % of freshly isolated liver macrophages spontaneously bind at this temperature senescent or in vitro aged RBC, binding of normal RBC is below 20 % (see legend Table 2). As shown in Table 2 the carbohydrate inhibition pattern of erythrocyte adhesion is identical to results obtained in the erythrophagocytosis assay (cf. Table 1). Furthermore there is a strict calcium dependence of RBC adhesion (Table 2). Inhibition studies with various concentrations of monosaccharides confirm that the order of inhibitory potency is GalNAc>Gal>> GlcNAc for recognition of senescent as well as of in vitro aged erythrocytes (Fig. 2).

Table 1 Carbohydrate-specific phagocytosis of senescent or *in vitro* aged erythrocytes (RBC) by liver macrophages

Saccharide (25 mM)	% Inhibition of phagocytosis (± S.D.) Senescent RBC (n = 3)	In vitro aged RBC (n = 4)
N-Acetyl-D-galactosamine	91.0 ± 6.3	77.0 ± 22.4
D-Galactose	61.5 ± 19.2	70.0 ± 15.3
L-Fucose	18.7 ± 16.4	11.3 ± 11.6
D-Glucose	32.7 ± 10.6	19.0 ± 17.8
D-Mannose	18.4 ± 16.0	19.9 ± 11.1
N-Acetyl-D-glucosamine	13.7 ± 12.7	29.2 ± 19.3

Carbohydrate inhibition experiments were performed by preincubation of adherent macrophages with saccharides for 1 min at 37°C followed by the addition of erythrocytes without removal of carbohydrates.

Table 2 Carbohydrate specificity of binding by liver macrophages of senescent and in vitro aged erythrocytes (RBC)

Inhibitor (25 mM)	% Inhibition of binding (± S.D.) Senescent RBC (n = 5)	In vitro aged RBC (n = 4)
N-Acetyl-D-galactosamine	81.2 ± 6.1	73.4 ± 13.1
D-Galactose	64.4 ± 13.7	65.1 ± 11.9
D-Fucose	68.2 ± 13.5	55.5 ± 3.4
L-Fucose	35.9 ± 14.2	22.7 ± 0.9
D-Glucose	22.8 ± 13.8	9.5 ± 7.6
D-Mannose	23.6 ± 10.6	26.0 ± 16.3
D-Fructose	24.9 ± 13.5	25.3 ± 5.5
N-Acetyl-D-glucosamine	24.0 ± 9.1	11.8 ± 6.6
EGTA + $MgCl_2$ (each 2.5 mM)	89.1 ± 4.1	88.4 ± 9.1

In the absence of inhibitors 45.5 ± 11.8 (n = 9) of liver macrophages bound senescent RBC and 50.3 ± 11.1 (n = 10) bound in vitro aged RBC. Binding of young RBC was 19.6 ± 10.7 (n = 9) and of freshly drawn RBC 17.5 ± 7.3 (n = 13). Inhibition studies were performed by incubation of liver macrophages with inhibitors for 5 min on ice prior to the addition of RBC.

Further inhibition experiments were carried out with synthetic glycoproteins, bovine serum albumin (BSA) conjugated with a number of different monosaccharides (14). Synthetic glycoproteins have been successfully used to study membrane lectins on mammalian cells (15-17). BSA conjugated with GalNAc (GalNAc-BSA) and Gal (Gal-BSA) turned out to be potent inhibitors of cell contacts between liver macrophages and senescent or in vitro aged RBC even at 10^{-8} to 10^{-10} M concentration (Table 3). Less inhibition is noted with Glc-BSA and L-Fuc-BSA, no inhibition with Man-BSA or GlcNAc-BSA. The difference in reactivity of GalNAc-BSA compared to GlcNAc-BSA by at least 5 orders of magnitude clearly shows that the recognition of senescent or in vitro aged RBC is carbohydrate-specific. These results led us to conclude that the putative receptor for old erythrocytes on liver macrophages is a GalNAc/Gal-binding membrane protein.

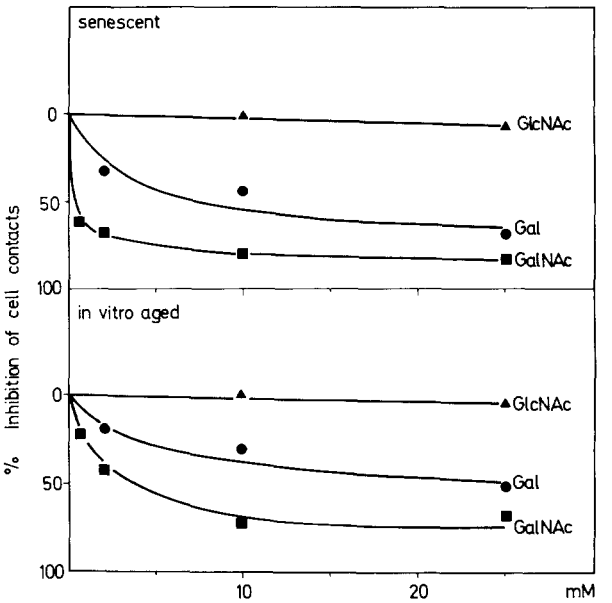

Fig.2 Inhibition of cell binding by various concentrations of monosaccharides. (A) Inhibition of adherence of senescent erythrocytes to liver macrophages, (B) inhibition of adherence of in vitro aged erythrocytes to liver macrophages, (GlcNAc) N-acetyl-D-glucosamine, (Gal) D-galactose, (GalNAc) N-acetyl-D-galactosamine. Data are the average of 2 experiments.

A survey of the literature shows that macrophages can express on the cell surface at least three different proteins with galactose binding capacity: (i) a 13-15 K protein found in many cell types including liver macrophages,

Table 3 Competitive inhibition of binding by liver macrophages of senescent or in vitro aged erythrocytes (RBC) by glycoproteins

Inhibitor	Concentration for 50 % inhibition (M)	
	Senescent RBC (n = 3)	In vitro aged RBC (n = 3)
GalNAc-BSA	3×10^{-10}	3×10^{-10}
Gal-BSA	4.9×10^{-9}	4.5×10^{-9}
Glc-BSA	1.1×10^{-8}	4.1×10^{-8}
L-Fuc-BSA	0.9×10^{-6}	2.5×10^{-6}
Man-BSA	$> 10^{-5}$	$> 10^{-5}$
GlcNAc-BSA	$> 10^{-5}$	$> 10^{-5}$
BSA	$> 10^{-5}$	$> 10^{-5}$

carbohydrate specifity: Lac >> Gal > GalNAc > D-Fuc, Man, GlcNAc, non calcium dependent (18, 19), (ii) the galactose-particle receptor described by several laboratories on macrophages of the liver and peritoneum, carbohydrate specifity: GalNAc > Gal, D-Fuc≫Man, GlcNAc, calcium dependent (20-25), and (iii) a galactose binding protein on peritoneal macrophages with carbohydrate specificity GalNAc >> Gal, D-Fuc, Man, GlcNAc, non calcium dependent (26). Of these three lectins the galactose-particle receptor is the only one that shares all characteristics described here for the recognition of old eryhtrocytes, i.e. the carbohydrate inhibition pattern GalNAc > Gal, D-Fuc≫Man, GlcNAc as well as the strict calcium dependence. The galactose-particle receptor has been previously called asialoglycoprotein receptor due to its close similarity in binding specificity to the analogous lectin on parenchymal liver cells (27,28). However, it has become clear that the galactose-particle receptor does not mediate endocytosis of soluble asialoglycoproteins (29,30) but only of particles or cells with asialoglycoproteins on the surface (20-25, 28).

Conflicting results have been reported about the nature of senescence-associated antigens on erythrocytes. Early speculations that sialidase-treated erythrocytes resemble old RBC (7,9) could not be supported by biochemical studies (12,13). However, as pointed out by Kay (4) the possibility of minor desialylation remains and is supported by two studies (11,12). A further finding in this direction is that autoantibodies eluted from senescent RBC bind to desialylated erythrocytes (5). Kay defined the autoantibody-reactive antigen on senescent and in vitro aged RBC as non band 3 protein (31), as a 72 K fragment of ankyrin (32), as a 62 K sialoglycoprotein (8) and recently as band 3 immunologically related polypeptides of 60 K, 42 K and 18 - 26 K molecular weight (33). Other laboratories have identified the senescent antigen as "polypeptide a" (210 K molecular weight) or as T-antigen carried by glycophorin A (34, 35).

From the experiments in this paper it appears that the determinants recognized by liver macrophages on age-specific antigens are galactose-related carbohydrates in terminal position. Whether these determinants reside on auto-

antibody-reactive antigens of senescent RBC or on other membrane constituents remains open. However, the reactivity of age-specific autoantibodies with desialylated RBC (5) suggests that they bind to ß-D-galactosyl residues uncovered by sialidase treatment. This would indicate that liver macrophages recognize the same determinants on senescent RBC as autoantibodies. We suggest that macrophages can recognize age-specific antigen(s) on old RBC by two mechanisms, either directly via a GalNAc/Gal-specific membrane receptor, or indirectly via the Fc-receptor and senescent cell-specific autoantibodies. The antibody mediated mechanism has been demonstrated by Kay (5) for monocytes. We have shown here, that macrophages of the liver, the physiological site of erythrocyte turnover, can bind and phagocytize old RBC without the help of antibodies.

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REFERENCES

1. Jenkin, C. R. and Karthigasu, K. (1962) Compt. Rend. Soc. Biol. 156, 1006-1007.
2. Bissell, D. M., Hammaker, L. and Schmid, R. (1971) Blood 38, 789.
3. Harris, J. W. and Kellermeyer, R. W. (1970) The Red Cell, pp. 526-561, Harvard University Press, Cambridge.
4. Kay, M. M. B. (1977) Liver and Ageing, pp. 87-109, Schattauer, Stuttgart.
5. Kay, M. M. B. (1975) Proc. Natl. Acad. Sci. USA 72, 3521-3525.
6. Murphy, J. R. (1973) J. Lab. Clin. Med. 82, 334-341.
7. Schlepper-Schäfer, J., Kolb-Bachofen, V. and Kolb, H. (1980) Biochem. J. 186, 827-831.
8. Kay, M. M. B. (1981) Nature 289, 491-494.
9. Baxter, A., and Beeley, J. G. (1975) Biochem. Soc. Trans. 3, 134-136.
10. Marikovsky, Y., Lotan, R., Lis, N., Sharon, N. and Danon, D. (1976) Exp. Cell Res. 99, 453-456.
11. Choy, Y. M., Wong, S. L. and Lee, C. Y. (1979) Biochem. Biophys. Res. Commun. 91, 410-415.
12. Platt, D. and Norwig, P. (1980) Mech. Ageing Dev. 14, 119-126.
13. Gattegno, L., Perret, G., Fabia, F. and Cornillot, P. (1981) Mech. Ageing Dev. 16, 205-219.
14. Lee, R. T. and Lee, Y. C. (1980) Biochemistry 19, 156-163.
15. Stowell, C. P. and Lee, Y. C. (1978) J. Biol. Chem. 253, 6107-6110.
16. Shepherd, V. L., Lee, Y. C., Schlesinger, P. W. and Stahl, P. (1981) Proc. Natl. Acad. Sci. USA 78, 1019-1022.
17. Kolb-Bachofen, V. (1981) Biochim. Biophys. Acta 645, 293-299.
18. Beyer, E.L., Tokuyasu, E. T. and Barondes, S. H. (1979) J. Cell Biol. 82, 565-571.
19. Harrison, F. L. and Chesterton, C. J. (1980) FEBS Lett. 122, 157-165.
20. Kolb, H. and Kolb-Bachofen, V. (1978) Biochem. Biophys. Res. Commun. 85, 678-683.
21. Nagamura, Y. and Kolb, H. (1980) FEBS Lett. 115, 59-62.

22. Küster, J. and Schauer, R. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1507-1514.
23. Kolb, H., Friedrich, E. and Süss, R. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1609-1614.
24. Müller, E., Franco, M.W. and Schauer, R. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1615-1620.
25. Perret, G., Vassy, R., Bladier, D., Gattegno, L. and Cornillot, P. (1982), Compt. Rend. Soc, Biol. 176, 266-271.
26. Nagamura, Y. and Kolb, H. (1981) Lectins - Biology, Biochemistry, Clinical Biochemistry, Vol. 1, pp. 33-42, de Gruyter, Berlin.
27. Kolb, H., Vogt, D., Herbertz, L., Corfield, A., Schauer, R. and Schlepper-Schäfer, J. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1747-1750.
28. Kolb-Bachofen, V., Schlepper-Schäfer, J., Vogell, W. and Kolb, H. (1982) Cell 29, 859-866.
29. Ashwell, G. and Morell, A.G. (1974) Adv. Enzymol. 41, 99-128.
30. Schlesinger, P. H., Doebber, T.W., Mandell, B. F., White, R., Deschryver, L., Rodman, J. S., Miller, M. J. and Stahl, P. (1978) Biochem. J. 176, 103-109.
31. Lutz, H. U. and Kay, M. M. B. (1981) Mech. Ageing Dev. 15, 65-75.
32. Kay, M. M. B.(1980) IXth International Symposium on Structure and Function of Erythroid Cells, pp. 12-15, Berlin.
33. Kay, M. M. B., Goodman, S. R., Sorensen, K., Whitfield, C. F., Wong, P., Zaki, L. and Rudloff, V. (1983) Proc. Natl. Acad. Sci. USA 80, 1631-1635.
34. Lutz, H. U. (1981) Acta Biol. Med. Germ. 40, 393-400.
35. Alderman, E. M., Fudenberg, H. H. and Lovins, R. E. (1981) Blood, 58, 341.