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Comparative quantitative analysis of macrophage populations defined by CD68 and carbohydrate antigens in normal and pathologically altered human liver tissue

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Abstract. Liver macrophages, which are involved in the different types of hepatitis, may indirectly induce hepatic fibrogenesis, since they have the possibility to activate hepatic stellate cells and fibroblasts by secretion of TGF-β, TNF-α and IL-1. To evaluate variations of the number of liver macrophages and their subpopulations, a quantification was carried out in normal human liver tissue, fatty liver, fatty liver hepatitis and hepatitis B. Identification was performed by the mab PG-M1 (anti-CD68) and, comparatively, four lectins, *Griffonia simplicifolia* agglutinin I (GSA-I), *Erythrina cristagalli* agglutinin (ECA), peanut agglutinin (PNA) and soybean agglutinin (SBA). A slight decrease in the frequency of macrophages in pericentral fields was observable in fatty liver and fatty liver hepatitis as compared to normal liver tissue. On the other hand, the number of $CD68^+$ cells was significantly enhanced in hepatitis B with moderate and severe inflammatory activity. The highest incidence of macrophages was found in portal tracts of liver with fatty liver hepatitis and, particularly, hepatitis B. The fraction of cells stained by ECA, PNA or SBA did not increase significantly under pathological conditions. In contrast, the percentage of GSA-I binding macrophages was higher in liver parenchyma of hepatitis B and in portal tract macrophages in fatty liver hepatitis and also hepatitis B. In conclusion, our results indicate that GSA-I may aid in the detection of the subpopulation of activated macrophages which are assumed to play a pivotal role in liver pathology.

Keywords: Kupffer cells, macrophages, CD68, lectins, *Griffonia simplicifolia*

1. Introduction

Human macrophages are an important part of the functional network of the immune system and are obviously divided into subpopulations representing different functional and activation states. Macrophages can easily and most specifically be identified by the monoclonal antibody (mab) PG-M1 [4], which is directed against a well-characterized mucin-type lysosomal glycoprotein, the CD68 antigen [9]. In liver pathology, ample evidence has been provided for an indirect role of macrophages

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in the development of fibrosis. For example, Kupffer cells secrete factors like TGF-β, TNF-α and IL-1, which activate hepatic stellate (fat-storing) cells and fibroblasts [1,3,5,6,14–16,26]. In a biliary fibrosis rat-model, a correlation of the numbers of hepatic stellate cells, Kupffer cells and the density of connective tissue was observed [7]. However, little information exists so far about the incidence of $CD68^+$ macrophages in normal and diseased liver tissue.

In the past years, renewed interest has been focused on liver macrophages which were characterized with respect to their expression of carbohydrate antigens. *Ulex europaeus* agglutinin I (UEA-I) and *soybean* agglutinin (SBA) were found to stain Kupffer cells in a fraction of liver specimens, whereas *peanut* agglutinin (PNA) was non-reactive with normal Kupffer cells, but showed a binding to portal macrophages and Kupffer cells in primary biliary cirrhosis, large duct obstruction and sclerosing cholangitis [20]. *Erythrina cristagalli* agglutinin (ECA) was also revealed to react with liver macrophages [23]. A specific staining of Kupffer cells and of interlobular vessels and bile duct structures by PNA, SBA, BPA (*Bauhinia purpurea* agglutinin) and GSA-I (*Griffonia simplicifolia* agglutinin I) was described by another group [17]. In contrast to these results, Yamamoto et al. [27] observed a reactivity of Kupffer cells in normal liver tissue with Concanavalin A, *Ricinus communis* agglutinin and *wheat germ* agglutinin, but not with PNA, SBA, UEA-I and DBA (*Dolichos biflorus* agglutinin). Additionally, in viral hepatitis, PNA displayed a specific binding to Kupffer cells. In a quantitative analysis of the relative frequency (percent area) of PNA positive Kupffer cells, an increase was disclosed ranging from chronic persistent to chronic aggressive hepatitis [27]. A comparison between the binding of CD68 specific mab and lectins to liver macrophages seems interesting, since the latter may detect different macrophage populations. In a recent study [2] we were able to establish that only a subpopulation of $CD68^+$ human bone macrophages expressed certain carbohydrate antigens detected by lectins like *Griffonia simplicifolia* agglutinin I isotype B4 (GSA-I-B4), *soybean* agglutinin (SBA) and *Erythrina cristagalli* agglutinin (ECA) as well as Le^a blood group antigen. In this context, it is noteworthy that numerous *in vitro* investigations using mouse and rat macrophages indicate that these macrophage subpopulations may represent activated cells [10,12,13, 18,21,24,25].

In the present study, serial sections of specimens from normal liver, fatty liver, fatty liver hepatitis and different grades of chronic hepatitis B were investigated. The total population of liver macrophages was visualized by the mab PG-M1 [4] and lectin histochemistry was performed for the identification and characterization of macrophage subpopulations.

2. Material and methods

2.1. Patients

Tissues from liver biopsies were derived from the files including 15 specimens from normal livers. The following non-viral liver diseases were entered into this study: fatty liver ($n = 15$), fatty liver hepatitis due to drug or alcohol toxicity ($n = 15$). Specimens from 28 patients known to be infected with hepatitis B virus (HBV) were scored histologically according to the classification of the International Association for the Study of the Liver (IASL): (1) no or minimal histological alteration ($n = 7$); (2) mild or moderate (score 2 or 3) inflammatory activity, fibrosis grade 2 or 3 ($n = 7$); (3) moderate inflammatory activity (score 2 or 3), cirrhosis ($n = 7$); (4) severe inflammatory activity (score 4), cirrhosis ($n = 7$).

Table 1
Biological origin and binding specificity of lectins applied in this study

	Origin	Monosaccharide(s) specificity	Oligosaccharide(s), blood group specificity
ECA	*Erythrina cristagalli*	αGalNAc	Galβ1-4GlcNAc
GSA-I	*Griffonia simplicifolia*	αGal	B blood group
PNA	*Arachis hypogaea*	α/βGal	Galβ1-3GalNAc (TF antigen)
SBA	*Glycine max*	α/βGal	GalNAcα1-3Galβ, A blood group

2.2. Monoclonal antibody and lectins

The mab PG-M1 (CD68) [4] was purchased from DAKO, Copenhagen, Denmark, and diluted 1 : 20 (v/v) in TBS/2.5% bovine serum albumin. *Erythrina cristagalli* agglutinin (ECA), *Griffonia simplicifolia* agglutinin I (GSA-I), *peanut* agglutinin (PNA) and *soybean* agglutinin (SBA) were purchased as biotinylated lectins from Medac, Hamburg, Germany (derived from E.Y. Lab., San Mateo, CA, USA), and applied in a dilution of 100 μg/ml (w/v) in the buffer mentioned above. Table 1 shows the binding specificities of these lectins.

2.3. Immunohistochemistry

Tissue specimens were fixed in 5% PBS-buffered formalin and embedded in paraffin. Five-micrometer thick sections were deparaffinized according to routine histological technique. It was established in preliminary experiments that proteolytic pretreatment of sections increased the reactivity of certain lectins, especially GSA-I and PNA [11]. Therefore, incubation with pronase E from *Streptomyces griseus* (1 mg/ml; Serva, Heidelberg, Germany) was performed for 30 min at 37°C. Monoclonal antibody PG-M1 as well as lectins were incubated overnight in a humid chamber at 4°C. The biotin–streptavidin–alkaline–phosphatase method was applied as described in detail elsewhere [2].

2.4. Immunohistochemical double-labelling experiments

Since it cannot be generally excluded that other cells stained by lectins may not be distinguished morphologically, we performed a double-labelling immunohistochemistry in order to determine the percentage of lectin-binding cells which are not PG-M1*. The following incubation steps were performed:

(1) biotinylated GSA-I (100 μg/ml), 60 min;
(2) strepatvidin–alkaline–phosphatase complex D396 (DAKO, Copenhagen, Denmark; 1 : 300), 30 min;
(3) Vector red substrate kit (Vector, Burlingame, CA, USA), 30 min;
(4) normal swine serum X901 (DAKO; 1 : 20), 30 min;
(5) PG-M1 (1 : 20), 60 min;
(6) rabbit anti-mouse immunoglobulin Z259 (DAKO; 1 : 50), 30 min;
(7) APAAP complex D651 (DAKO; 1 : 50), 30 min;
(8)/(9) repeating of steps (6) and (7);
(10) Vector blue substrate kit (Vector).

All incubation steps were carried out at room temperature. After every step – with the exception of step (4) – three-fold washing with TBS was performed. Vector kits were applied according to the manufacturer's description. As a control, steps (1) and (2) were replaced by steps (5)–(9) and vice versa, using PG-M1 or GSA-I, respectively, in both staining steps.

2.5. Morphometry and statistical evaluation

Generally, macrophages were counted per square millimetre (mm^2) at a magnification of $400\times$ using a grid ocular. Pericentral and portal fields were evaluated separately. Macrophages in portal fields from normal livers, fatty livers and HBV-infected livers without inflammatory activity or cirrhosis were counted per square millimeter using a manual optic planimeter (MPO-A-MO1-Kontron) involving a standard program set (Kontron software), as described earlier [2]. Only cells containing a nucleus stained by haematoxylin were considered. Endothelial cells as well as bile duct cells reactive with some lectins were excluded. Counting was performed without knowledge of the diagnosis of the specimen by two observers independently. The difference of their results was never greater than 10%, and the mean value was used. The statistical significance of differences between two groups of data was calculated using the Mann–Whitney U-test.

3. Results

3.1. Quantification of CD68 positive macrophages

In normal liver, Kupffer cells were distinctively stained by PG-M1 (155 ± 24 cells/mm^2) (Fig. 1(a)), but also showed a certain reactivity with all lectins under study. In specimens of fatty liver (Table 2), the number of macrophages was decreased (129 ± 27), possibly due to the enhanced volume of fat-storing hepatocytes. On the opposite, the number of macrophages stained by PG-M1 in the pericentral fields of fatty liver hepatitis was generally higher than that in fatty liver, but did not exceed the value of the normal liver. For the details of morphometric analysis, see Table 2. Pericentral fields of biopsies derived from patients suffering from chronic HBV infection but without accompanying florid hepatitis revealed a number of PG-M1 stained macrophages ($159 \pm 29/mm^2$) which was similar to normal liver tissue. In comparison, HBV-infected livers showing moderate inflammatory activity not accompanied by cirrhosis (Table 2) exhibited a significantly ($p < 0.005$) elevated number ($285 \pm 63/mm^2$) of PG-$M1^+$ macrophages in the pericentral fields. However, the frequency was lower in pericentral fields of livers with moderate inflammatory activity and cirrhosis. Incidence increased again significantly ($p < 0.01$) in cases with severe inflammation (Fig. 1(d); Table 2). In the portal tracts of fatty liver hepatitis (Table 3), the lymphohistiocytic infiltration was associated with a high number of macrophages compared to normal and fatty livers as well as HBV-infected livers without inflammatory activity. A significantly ($p < 0.05$) higher amount of reactive macrophages per mm^2 (1173 ± 373), however, was observed in the portal tracts of chronic hepatitis B exhibiting moderate inflammatory activity without cirrhosis (Table 3). Accompanying the development of cirrhosis, the number of macrophages in the portal tracts decreased to slightly lower values which was possibly due to the space occupated by connective tissue.

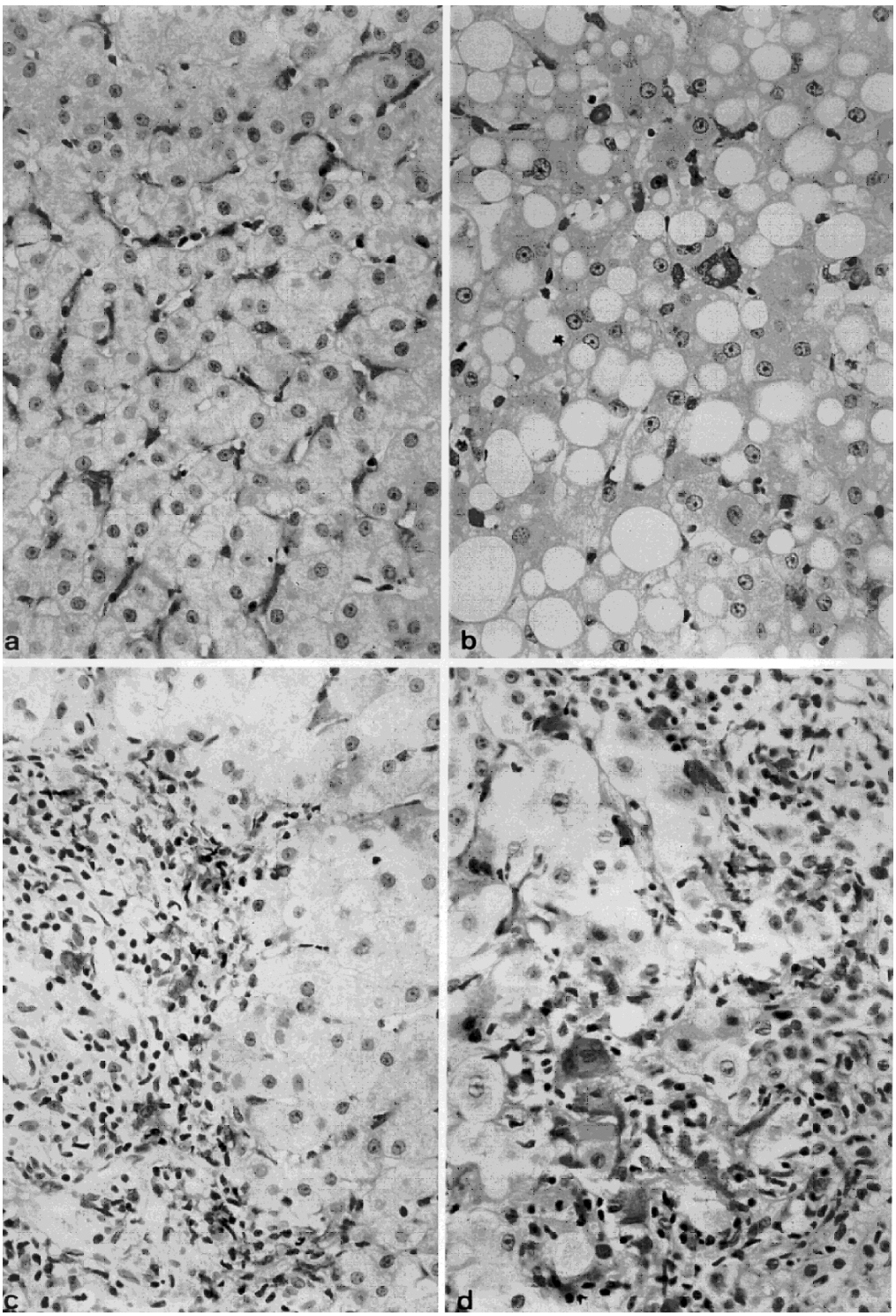

Fig. 1. PG-M1 staining of normal (a) and fatty (b) liver reveals macrophages in the pericentral fields. In hepatitis B with moderate (c) and severe (d) inflammation, the number of macrophages is markedly elevated. Original magnification ×570.

Table 2
Morphometric analysis of macrophages in the pericentral fields of normal and fatty livers, fatty liver hepatitis and HBV-infected livers (ranges, medians, means and standard deviations)

	PG-M1	ECA	GSA-I	PNA	SBA
	(number of cells stained per mm^2)				
	Normal liver ($n = 15$)				
Range	101–197	87–192	18–63	75–138	40–162
Median	158	128	30	109	113
Mean	155	129	33	107	109
Standard deviation	24	26	11	17	34
	Fatty liver ($n = 15$)				
Range	88–175	64–135	22–70	62–111	46–141
Median	128	106	33	77	81
Mean	129	103	37	81	93
Standard deviation	27	18	14	15	29
	Fatty liver hepatitis ($n = 15$)				
Range	81–261	60–159	25–126	45–143	58–214
Median	134	120	45	86	98
Mean	161	115	51	90	109
Standard deviation	58	28	24	31	46
	No/minimal inflammatory activity, no cirrhosis ($n = 7$)				
Range	122–207	97–150	28–72	50–105	53–146
Median	158	138	56	92	113
Mean	159	131	52	84	108
Standard deviation	29	20	16	19	33
	Moderate inflammatory activity, no cirrhosis ($n = 7$)				
Range	171–345	123–195	48–142	33–169	93–229
Median	310	169	78	135	164
Mean	285	162	85	128	157
Standard deviation	63	22	36	42	40
	Moderate inflammatory activity, cirrhosis ($n = 7$)				
Range	127–290	109–221	24–91	91–125	38–131
Median	193	143	55	114	105
Mean	197	164	53	112	98
Standard deviation	50	44	21	12	27
	Severe inflammatory activity, cirrhosis ($n = 7$)				
Range	248–425	160–384	27–193	73–266	160–322
Median	309	211	123	145	185
Mean	314	248	119	160	206
Standard deviation	58	84	52	67	53

3.2. Quantification of lectin-binding cells

The number of lectin-binding cells was correlated with the number of PG-M1$^+$ macrophages (Table 4). Our results show that this relation is not constant in normal and pathologically altered liver tissue. However, the number of cells stained by the various lectins never exceeded the number of PG-M1$^+$ cells. The relative number of ECA$^+$, PNA$^+$ and SBA$^+$ cells in fatty liver and fatty liver hepatitis did not change remarkably compared to normal tissue. In HBV-infected livers, ECA, PNA and SBA identified an equal or even higher percentage of macrophages in the pericentral fields without or with minimal histological changes compared to those exhibiting moderate or severe inflammation.

Table 3
Morphometric analysis of macrophages in the portal fields of normal and fatty livers, fatty liver hepatitis and HBV-infected livers (ranges, medians, means and standard deviations)

	PG-M1	ECA	GSA-I	PNA	SBA
	(number of cells stained per mm^2)				
	Normal liver ($n = 9$)				
Range	49–195	29–152	12–75	29–141	29–172
Median	95	65	22	58	81
Mean	109	70	32	66	83
Standard deviation	44	36	21	31	38
	Fatty liver ($n = 5$)				
Range	200–334	158–211	101–211	135–202	146–214
Median	266	200	140	155	177
Mean	264	192	143	159	175
Standard deviation	53	19	39	25	25
	Fatty liver hepatitis ($n = 15$)				
Range	496–1233	308–755	152–404	90–614	304–729
Median	710	491	285	330	539
Mean	784	492	285	347	513
Standard deviation	229	130	83	143	121
	No/minimal inflammatory activity, no cirrhosis ($n = 5$)				
Range	244–353	157–298	106–233	113–240	111–191
Median	259	193	135	156	154
Mean	290	209	161	165	156
Standard deviation	49	48	50	47	28
	Moderate inflammatory activity, no cirrhosis ($n = 7$)				
Range	684–1830	309–832	320–656	308–678	585–848
Median	1109	575	415	499	640
Mean	1173	595	473	492	695
Standard deviation	373	179	122	126	98
	Moderate inflammatory activity, cirrhosis ($n = 7$)				
Range	835–1181	252–667	191–341	209–442	353–727
Median	896	554	258	339	459
Mean	971	497	267	333	492
Standard deviation	128	145	51	78	112
	Severe inflammatory activity, cirrhosis ($n = 7$)				
Range	656–1114	167–630	228–468	92–494	406–724
Median	837	410	293	378	533
Mean	828	421	318	318	526
Standard deviation	141	137	81	140	93

Moreover, the percentage of ECA and PNA binding cells in the portal tracts in fatty liver hepatitis is lower than in the pericentral fields. Altogether, the portion of ECA$^+$ and PNA$^+$ cells in relation to CD68$^+$ macrophages was not increased in hepatitis B specimens containing inflammation and/or cirrhosis compared to normal livers, fatty liver and also fatty liver hepatitis.

3.3. Percentage of GSA-I positive macrophages

In comparison with normal liver tissue, the portion of GSA-I$^+$ cells in the pericentral fields increased significantly ($p < 0.05$) during the evolution of fatty liver. An even higher value was encountered in

Table 4
Means of numbers of lectin-positive cells expressed as percentage of the mean number of $CD68^+$ macrophages

	ECA		GSA-I		PNA		SBA	
	PC	POR	PC	POR	PC	POR	PC	POR
Normal liver	83	64	21	29	69	61	70	76
Fatty liver	80	73	29	54	63	60	72	66
Fatty liver hepatitis	71	63	32	36	56	44	68	65
HBV: no inflammation, no cirrhosis	82	72	33	56	53	57	68	54
HBV: mod. inflammation, no cirrhosis	57	51	30	40	45	42	55	59
HBV: mod. inflammation, cirrhosis	83	51	27	27	57	34	50	51
HBV: severe inflammation, cirrhosis	79	51	38	38	51	38	66	64

PC = pericentral fields; POR = portal fields.

the pericentral fields and portal tracts of specimens with fatty liver hepatitis (Table 3). Contrasting PG-M1 and ECA, which stained nearly equal numbers of cells in normal and HBV-infected livers without relevant pathological changes, GSA-I reacted with 56 ± 16 cells per mm^2 in the pericentral fields of the latter specimens compared to 30 ± 11 cells in normal liver biopsies ($p < 0.05$). In the portal tracts of fatty liver, fatty liver hepatitis and HBV-infected liver, the number of GSA-I^+ macrophages was increased (Table 3). In summary, the portion of GSA-I^+ cells showed an increase in all forms of inflammatory liver disease as compared to normal liver tissue (Table 4).

3.4. Control: Immunohistochemical double-labelling

Double-labelling control experiments using PG-M1 and GSA-I were performed staining 3 specimens representing each diagnosis included in the study. The percentage of red cells, i.e., cells without double-staining by PG-M1, which resulted in a blue–red mixture or pure blue staining, did not exceed 7%. The control sections did not show a blue staining reaction, indicating that cross-reactivities of Vector blue with antigens bound in step (1), did not contribute to the Vector red staining reaction.

4. Discussion

4.1. Macrophages in liver diseases

In the present study, the number of macrophages as detected by the CD68 specific mab PG-M1 in normal liver parenchyma and various liver diseases was evaluated. Consequently, our findings are in agreement with recently published data on other liver cell populations like α-SMA^+ Ito cells [19]. An increase of PG-$M1^+$ macrophages could be particularly observed in HBV-induced hepatitis with moderate and severe inflammatory activity. The highest frequencies of macrophages, however, were obtained in the portal tracts of livers exhibiting fatty liver hepatitis or HBV infection. Using an experimental rat model, a correlation between the numbers of hepatic stellate as well as Kupffer cells with evolution of fibrosis following bile duct ligation has been observed [7,8].

4.2. Characterization of liver macrophages by CD68 antigen and lectin-binding sites

Comparatively, four lectins (GSA-I, ECA, PNA, SBA) reported previously [17,20,23,27] to identify liver macrophages were investigated. However, these stained varying numbers of cells, which were always lower than the number of PG-$M1^+$ macrophages. Therefore, it is reasonable to assume that all

of them detect only subpopulations of liver macrophages. On the other hand, it cannot be definitively ruled out by light microscopical immunohistochemistry that all cells stained by these lectins are really macrophages. Whereas endothelial and bile duct cells may easily be excluded from counting. It may be diffcult to discriminate between myofibroblastic or hepatic stellate cells. For this reason, lectins are a less reliable tool for the identification of liver macrophages compared to a CD68-specific mab. Furthermore, our results are not in agreement with the hypothesis of Yamamoto et al. [27], who regarded PNA to be a marker of activated Kupffer cells, since it did not stain Kupffer cells in normal liver parenchyma. However, in their study, the number of PNA^+ cells was not counted but quantified by image analysis as percent area of the parenchyma. Inherent to this method, enlargement of cells and variations of staining intensity may significantly influence the results and therefore a number of caveats have to be taken into account when interpretating the findings of these authors.

4.3. GSA-I binding sites on activated macrophages

Ample evidence has shown that the mab PG-M1 directed against the CD68 antigen can be regarded as the most specific tool for the visualization of liver macrophages. The use of three lectins in this study (ECA, PNA, SBA) does not result in relevant information additional to that obtained by PG-M1 staining. However, the reactivity pattern of GSA-I lectin suggests that not only the number of macrophages, but also the percentage of the activated subfraction may increase in the course of inflammatory activity as well as with periportal fibrogenesis. As reported in several experimental studies, GSA-I characterizes a carbohydrate antigen associated with macrophage activation [10,13,18, 21,24,25]. By double-labelling immunohistochemistry could be ruled out that a significant portion of $GSA\text{-}I^+$ cells in human liver do not belong to the $CD68^+$ macrophage lineage. The percentage of $GSA\text{-}I^+$ cells related to the number of $PG\text{-}M1^+$ macrophages was significantly increased in the pericentral fields in inflammatory liver disease as compared to normal livers. A high portion of $GSA\text{-}I^+$ cells was also observed in the portal tracts under these conditions, which may also be regarded as activated macrophages. Interestingly, *in vivo* studies [22] have shown that binding of GSA-I-B4 to peritoneal mice macrophages induces the secretion of TNF-α, a mediator which is involved in hepatic fibrogenesis like TGF-β and IL-1 [1,3,5,6,14–16,26]. Considering these findings and the results of the present study it is tempting to speculate that not only the absolute number of Kupffer cells, but their expression of α-D-galactose containing glycoproteins are part of the complex functional cellular network inducing hepatic fibrogenesis. For this reason, our results are in keeping with the assumption that the $GSA\text{-}I^+$ activated macrophage subpopulation plays a key role in liver pathology.

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