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Characterization of the binding specificity of *Anguilla anguilla* agglutinin (AAA) in comparison to *Ulex europaeus* agglutinin I (UEA-I)

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Using immunochemical and immunohistochemical methods, the binding site of *Anguilla anguilla* agglutinin (AAA) was characterized and compared with the related fucose-specific lectin from *Ulex europaeus* (UEA-I). In solid-phase enzyme-linked immunoassays, the two lectins recognized Fucα1-2Galβ-HSA. AAA additionally cross-reacted with neoglycolipids bearing lacto-*N*-fucopentaose (LNFP) I [H type 1] and II [Le^a] and lactodifucotetraose (LDFT) as glycan moieties. UEA-I, on the other hand, bound to a LDFT-derived neoglycolipid but not to the other neoglycolipids tested. Binding of AAA to gastric mucin was competitively neutralized by Le^a-specific monoclonal antibodies. UEA-I binding, on the other hand, was reduced after co-incubation with H type 2- and Le^y-specific monoclonal antibodies. According to our results, AAA reacts with fucosylated type 1 chain antigens, whereas UEA-I binds only to the α1-2-fucosylated LDFT-derived neoglycolipid. In immunohistochemical studies, the reactivity of AAA and UEA-I in normal pyloric mucosa from individuals with known Lewis and secretor status was analysed. AAA showed a broad reaction in the superficial pyloric mucosa from secretors and non-secretors, but AAA reactivity was more pronounced in Le$^{(a+b-)}$ individuals. On the other hand, UEA-I stained the superficial pyloric mucosa only from secretor individuals. A staining of deep mucous glands by the lectins was found in all specimens. Both reacted with most human carcinomas of different origin. Slight differences in their binding pattern were observed and may be explained by the different fine-specificities of the lectins.

Keywords: lectin, *Anguilla anguilla* agglutinin, carbohydrate antigen, tumour-associated antigen

Introduction

Various 'fucose-specific' lectins derived from different sources are important reagents in glycobiology, histo- and cytochemistry [1–3]. In 1952, Watkins and Morgan demonstrated [4] that the agglutination of blood group O and A_2 erythrocytes exerted by a substance derived from the serum of *Anguilla anguilla* can be inhibited by α-methyl-L-fucopyranoside and L-fucose. Contrasting this finding, other monosaccharides, especially D- and L-glucose, D- and L-galactose and *N*-acetyl-D-galactosamine failed to inhibit this reaction. Further studies regarding its binding to fucose and corresponding derivatives followed [5–7]. An additional capacity to detect terminal L-galactose residues could be shown by the use of various galactan polysaccharides [8]. However, a further characterization of its fine specificity, i.e. affinity towards α1-2fucosylated type 1 or type 2 backbones and related Lewis antigens was not performed. On the other hand, related 'fucose-specific' lectins like *Ulex europaeus* agglutinin I (UEA-I) [9–12] and *Lotus tetragonolobus* agglutinin (LTA) [13, 14] were extensively characterized by immunochemical methods. According to these studies, UEA-I preferentially binds to Fucα1-

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2Galβ1-4GlcNAc (H type 2 blood group antigen) with a cross-reactivity to Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ (Lewis^y antigen), but not to internal α-L-fucose units. LTA, on the other hand, is most strongly inhibited by Fucα1-6GlcNAc, followed by Fucα1-2Galβ1-4(Fucα1-3) GlcNAcβ. We recently observed striking differences in the cyto- and histochemical reaction to human megakaryocytes exerted by UEA-I on the one side and AAA and LTA on the other hand [15]. Similar discrepancies were also reported by Ito *et al.* in their study [16] on lectin binding to ABH antigens in other human tissues. Therefore, the purpose of this study was: (1) to characterize the binding specificity of AAA by immunochemical analysis in comparison to the well-investigated lectin UEA-I; and (2) to evaluate their immunohistochemical staining pattern in gastric tissues defined by secretor and Lewis status as well as carcinomas originating from various organs.

Materials and methods

MATERIALS

Biotinylated *Anguilla anguilla* agglutinin (AAA) as well as biotinylated and unlabelled *Ulex europaeus* agglutinin 1 (UEA-I) were purchased from E-Y Laboratories (San Mateo, California, USA). Monoclonal antibodies (mabs) 12-4LE [17], 19-0LE (cross-reacting between H type 2 and Le^y) [18, 19], 7-LE (anti-Le^a) [19] and 121-SLE (anti-sialosyl-Le^a) were generated in the laboratory of J. Bara. Their specificities were extensively studied previously [20]. Mab Bw494 detecting a terminal Galβ1-3HexNAc unit and cross-reacting between Le^a and Tβ antigen [21] was a kind gift from Behringwerke (Marburg, Germany). Monoclonal antibody LeuM1 (anti-Le^x) was from Becton-Dickinson (Heidelberg, Germany). Fucα1-2Galβ1-0(CONH)$_{30}$-HSA was a kind gift from Dr Kolar (Behringwerke, Marburg, Germany). The following oligosaccharides derived from human milk were purchased from Oxford Glycosystems (Abingdon, Oxon, United Kingdom):

1. Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LNFP I) [H type 1],
2. Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc (LNFP II) [Le^a],
3. Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc (LNDFH I) [Le^b],
4. Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc (LNFP III) [Le^x],
5. Fucα1-2Galβ1-4(Fucα1-3)Glc (LDFT),
6. Galβ1–3GlcNAcβ1-3Galβ1-4Glc (LNT).

Gastric juice was collected and pooled according to the secretor and Lewis phenotype of the donors. Mucus glycoproteins were isolated following previously published procedures [22] by extraction with 90% phenol.

Preparation of neoglycolipids

Neoglycolipids of LNFP I, LNFP II, LNFP III, LNDFH I and LDFT were prepared according to Stoll *et al.* [23] by coupling to dipalmitoylglycerophosphoethanolamine via reductive amination.

Immunochemical analysis

Antigens diluted in 0.1 M carbonate buffer, pH 9.6 (glycoproteins) or in 40% aqueous methanol (neoglycolipids) were coated to 96-well polystyrene microtitre plates (Nunc, Wiesbaden, Germany) at 37 °C overnight. After blocking of unspecific binding sites with PBS (20 mM phosphate, 0.15 M NaCl, pH 7.2) containing 5% bovine serum albumin (BSA, Sigma, Munich, Germany) for 1 h at 37 °C, biotinylated lectins were incubated for 2 h at room temperature. All washing steps and lectin dilutions (to 10 $\mu g\,ml^{-1}$) were performed with PBS/0.5% BSA. Streptavidin-alkaline-phosphatase conjugate, diluted 1:5000, was incubated for 30 min at room temperature. The reaction was developed with p-nitrophenylphosphate (1 $mg\,ml^{-1}$) in 50 mM diethanolamine buffer pH 9.8 containing 0.5 mM $MgCl_2$ (30 min, 22 °C).

Competition assays with mabs 12-4LE, 19-0LE, 7-LE, 121-LE, Bw494 and LeuM1 and unlabelled UEA-I were carried out on gastric mucins pooled from Le$^{(a+b-)}$ individuals (25 $\mu g\,ml^{-1}$) with AAA and UEA-I in a dilution of 10 $\mu g\,ml^{-1}$ Mabs were used as undiluted cell culture supernatants (12-4LE, 19-0LE, 7-LE, 121-SLE), at 50 $\mu g\,ml^{-1}$ (Bw494) or 1:10 (by vol) (LeuM1), respectively. Non-biotinylated UEA-I was employed at 50 $\mu g\,ml^{-1}$. AAA and mabs were co-incubated for 1 h at 37 °C.

Immunohistochemical labelling

Human carcinoma tissues were derived from the files of the Institute of Pathology at the University of Cologne. Normal pyloric tissues were obtained from kidney donors with proven brain death and provided from the Centre de Recherches Paris Saint-Antoine. These specimens were fixed with 95% ethanol and defined with regard to their secretor and Lewis status [20]. Human tissues were fixed in 5% neutral-phosphate buffered formalin and embedded in paraffin. Five μm thick sections were cut and deparaffinized following standard histological techniques. After blocking of endogenous peroxidase using 1% H_2O_2 in methanol for 30 min, slides were incubated with biotinylated lectins (100 μg per ml TBS per 2.5% BSA) for 1 h. Specimens were incubated with streptavidin-peroxidase complex P396 (Dakopatts, Copenhagen, Denmark, diluted 1:400 in TBS per 2.5% BSA) for 1 h. Between all steps, which were performed at room temperature, the slides were threefold washed in TBS. The reaction was developed by 200 $\mu g\,ml^{-1}$ (w/v) 3-amino-9-ethyl-carbazole (Sigma, Munich, Germany) in

0.05 M sodium acetate buffer containing 5% dimethylformamide and 0.01% H_2O_2 for 30 min at room temperature. After counterstaining with Hematoxylin (5 min), the slides were mounted in glycerol jelly.

Binding specificity of AAA and UEA-I was controlled by co-incubation with 100 mM α-L-fucose.

Results and discussion

Immunochemical characterization

In direct binding studies, AAA and UEA-I strongly bound to Fucα1-2Galβ-HSA (Fig. 1). Binding of AAA and UEA-I to various neoglycolipids derived from milk oligosaccharides showed marked differences in the corresponding patterns of these lectins: AAA detected LNFP I (bearing H type 1 antigen), more weakly also LNFP II, i.e. Le^a antigen, but did not react with LNFP III or LNT (Fig. 2a). Such a binding pattern implies a reactivity with particular type 1 oligosaccharide structures. The α1-2-fucosylated LDFT, which is structurally modified by reductive amination, was bound by AAA and UEA-I. All other neoglycolipids failed to display a significant reactivity with the latter lectin (Fig. 2b). This finding corresponds to the observation that UEA-I exerts a strong reactivity only with H type 2 and Le^y antigen [10–12, 19], but not with type 1 oligosaccharides. Our results suggest the conclusion that AAA differs from UEA-I by a strong binding to H type 1 as well as Le^a.

Binding to gastric mucins from $Le^{(a+b-)}$ and $Le^{(a-b+)}$ individuals revealed further characteristic differences: AAA showed the same strength of reactivity towards both, whereas UEA-I exhibited a clear preference for the $Le^{(a-b+)}$-derived mucin, i.e. α1-2-fucosylated glycoprotein (Fig. 3a,b).

When the competitory activity of mabs and UEA-I towards AAA and UEA-I binding to gastric mucin from $Le^{(a+b-)}$ individuals was tested, AAA reactivity was markedly reduced by Le^a-specific mabs 7-LE [19] and BW494 [22] (Table 1). UEA-I binding, on the other hand, was strongly inhibited competitively by UEA-I and mabs

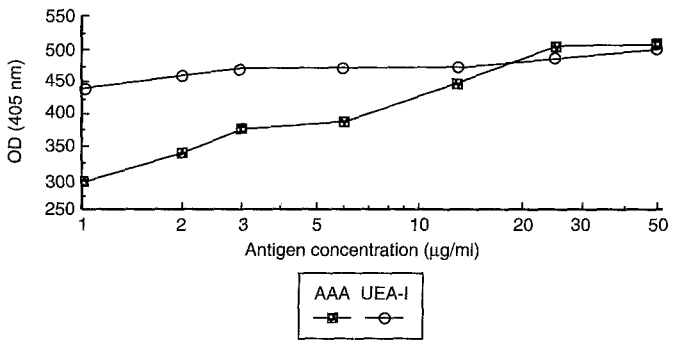

Figure 1. Binding activity of AAA and UEA-I (10 $\mu g\,ml^{-1}$) to Fucα(1-2)Galβ-HSA measured by ELISA.

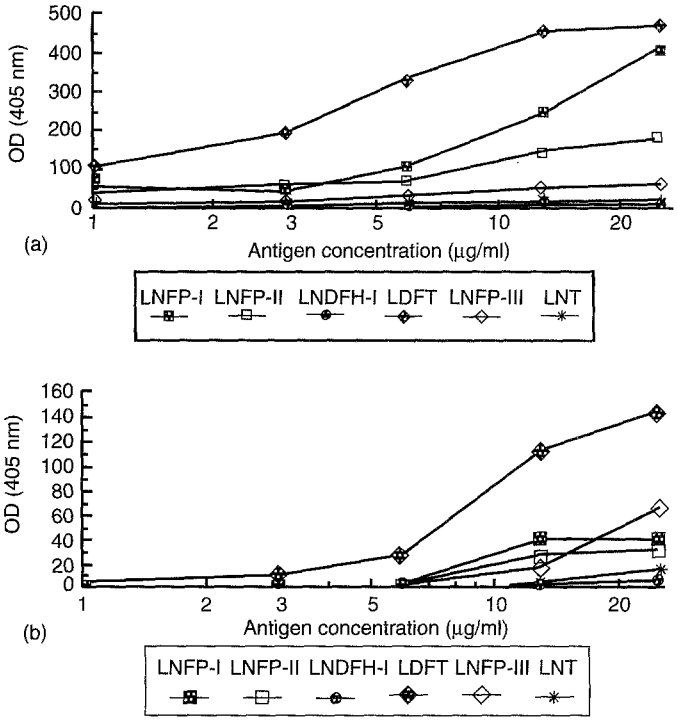

Figure 2. ELISA reactivity of (a) AAA, 10 $\mu g\,ml^{-1}$, and (b) UEA-I, 10 $\mu g\,ml^{-1}$, with different neoglycolipids synthesized according to Materials and methods.

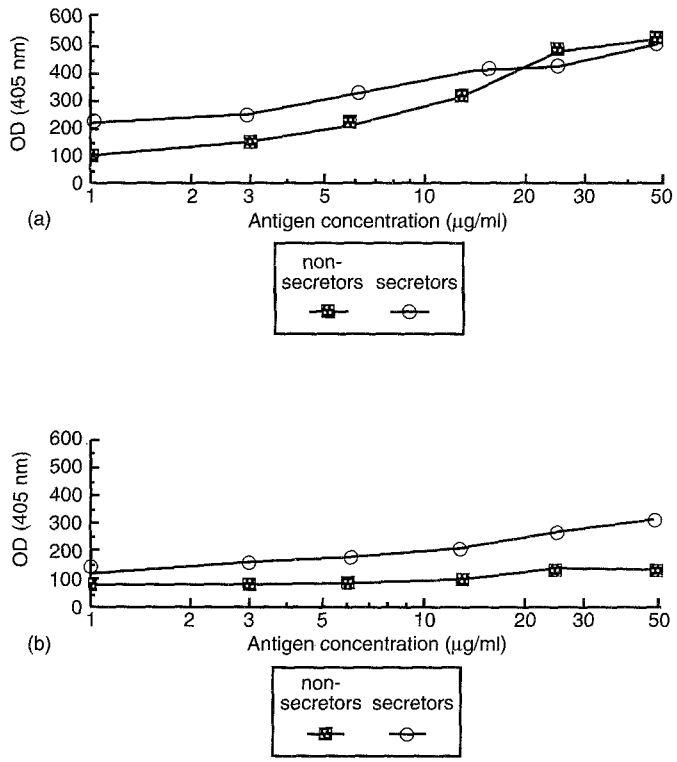

Figure 3. Binding of AAA and UEA-I (10 $\mu g\,ml^{-1}$) to gastric mucins pooled according to the secretor status of the donors tested by ELISA. (a) AAA (b) UEA-I.

Table 1. Competitive inhibition of AAA and UEA-I binding to gastric mucin from $Le^{(a+b-)}$ individuals by various mabs and UEA-I.

Competitor	*Binding of AAA*	*UEA-I*
Bw494	0.44	0.75
7-LE	0.26	0.78
121-SLE	0.64	0.71
UEA-I	0.74	0.42
19-0LE	0.84	0.65
12-4LE	0.86	0.47
LeuM1	0.84	0.81

This Table shows the binding activity of AAA and UEA-I retained after co-incubation with the competitors indicated. Binding without inhibitor, 1.0.

Table 2. Reactivity of AAA and UEA-I with pyloric surface epithelium.

ABH and Lewis blood group antigen expression	n	*Staining pattern of*	
		AAA	*UEA-I*
ALe^a	1	++/+	0
OLe^a	3	++/+	0
ALe^b	3	+/++	++/+
BLe^b	1	+/++	++/+
OLe^b	3	+/++	++/+

Scoring: 0, no reactivity; +, low reactivity; ++, strong reactivity. Where two scores are given, the first score indicates the staining intensity seen in the majority of the samples. n, number of specimens investigated.

12-4LE and 19-0LE which detect H type 2 and Le^y antigens (Table 1). All the other mabs tested exerted only minimal competitive effects.

Our immunochemical investigation reveals that AAA binds to particular type 1 antigens (H type 1, Le^a) in direct binding assays. A significant competitive effect on lectin binding to gastric mucins is only exerted by Le^a-specific mabs. Opposed to this finding, like UEA-I, as has been described in previous reports [11] and in this study, neither LTA nor *Galactia tenuiflora* agglutinin [19] showed any binding to Le^a. The latter differs from UEA-I since it has a certain affinity to H type 1, but does not bind difucosylated structures [19, 24, 25]. UEA-I and LTA, on the other hand, did not exert a relevant reactivity with H type 1 antigen [11, 19]. Such differences may be interpreted by the dependence of lectin binding to antigenic determinants from certain chemical conformations [26]. In conclusion, AAA has a carbohydrate binding specificity which is distinct from other 'fucose-specific' lectins.

Immunohistochemical studies

Immunohistochemical characterization of AAA and UEA-I was performed on malignant and benign epithelial tissues from different organ sites. The reactivity of normal pyloric mucosa with known secretor and Lewis status was tested (Table 2). Briefly, AAA stained the surface epithelium in all specimens under study (Fig. 4a). However, the binding was more intense in those derived from $Le^{(a+b-)}$ individuals. On the other hand, binding of UEA-I towards surface epithelium was only seen in specimens derived from individuals expressing the secretor gene ($Le^{(a-b+)}$). Pyloric mucous glands were stained by AAA and UEA-I without correlation to the secretor or Lewis properties (Fig. 4b). These observations fit in well with the results of Mollicone *et al.* [27, 28], who established that biosynthesis of α1-2-fucosylated antigens in the surface epithelium can only be observed in secretors, whereas such antigens are expressed in pyloric mucous glands independent from secretor status.

Table 3. Reactivity of AAA and UAE-I with human carcinomas.

Origin	n	*Number stained by*	
		AAA	*UEA-I*
Gastric	15	12	11
Duodenal (papillary)	1	1	1
Colorectal	2	2	2
Pancreatic	2	1	0
Lung	3	1	2
Mammary	4	2	3
Prostatic	3	2	2
Renal, trans. cell	2	0	0
Ovarian	4	3	2

n, number of specimens tested.

Carcinomas of the stomach, colorectum, pancreas, lung and mammary gland were tested to obtain data on the possible alterations of lectin binding sites in malignant tissues. AAA and UEA-I reacted with the vast majority of these carcinomas. The results are summarized in Table 3. Membraneous as well as cytoplasmic and secretion-bound staining was observed (Fig. 4c–e). Comparison of the binding patterns in the individual carcinomas revealed differences between the two lectins. As could be expected when taking into account the immunochemical analysis, UEA-I binding was more distinct compared to AAA binding. It had to be expected that most carcinomas are reactive with fucose-specific lectins, since the expression of ABH and Lewis antigens is a typical event in carcinogenesis [29–31]. However, immunochemical analysis as well as immunohistochemical demonstration of carbohydrate antigens *in situ* depend on the exact knowledge of the fine specificities of the lectins or monoclonal antibodies used.

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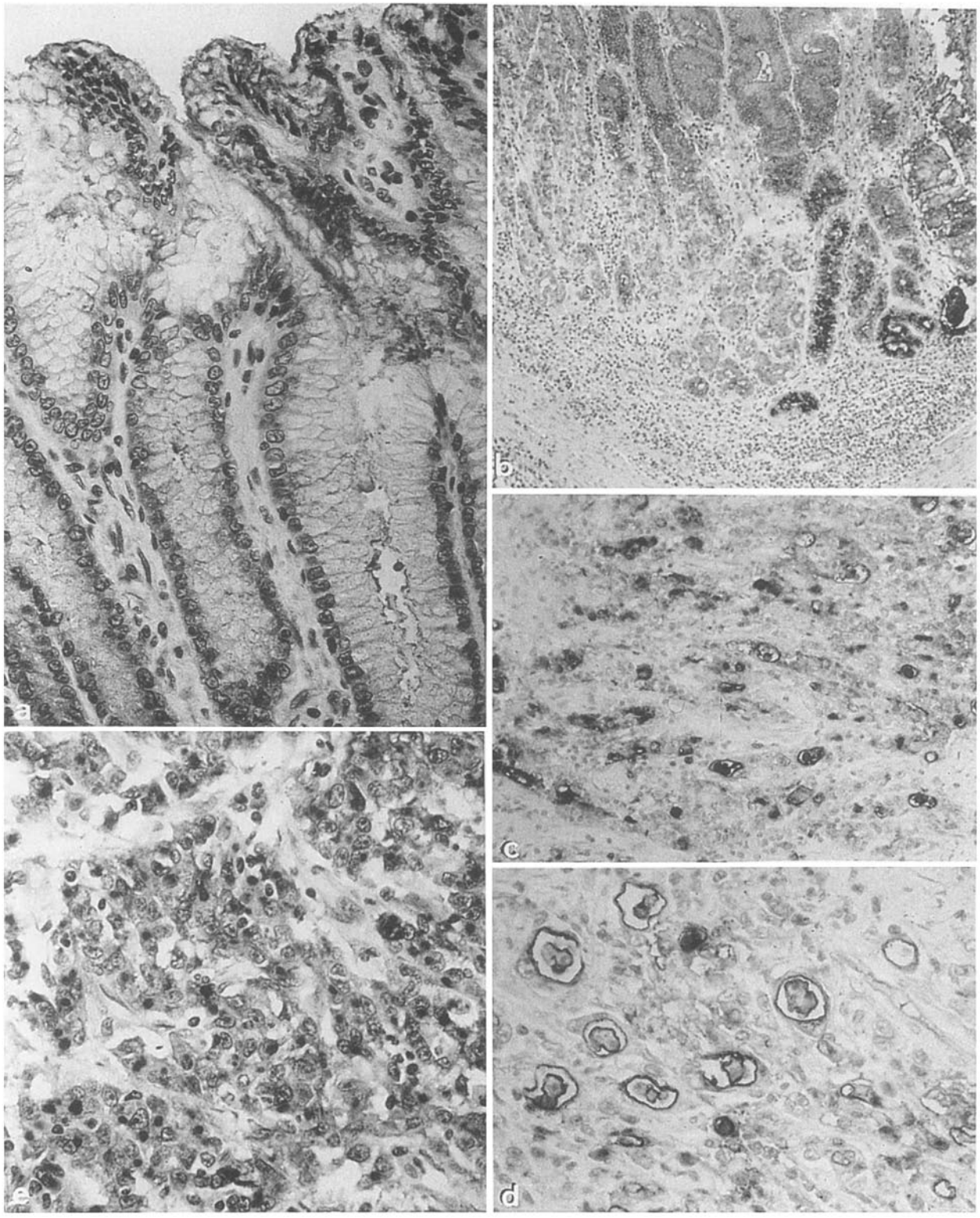

Figure 4. Immunohistochemical demonstration of lectin binding sites. Binding of AAA to (a) pyloric surface epithelium as well as (b) mucous glands is independent from the secretor status of the donors. Gastric carcinomas (c–e) show a strong staining by AAA, which may be associated to membranes or secretions of tumour cells or to perinuclear cytoplasmic structures. Magnification ×350 (a, d, e) or ×90 (b, c), respectively.

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