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PNA-binding glycans are expressed at high levels on horse mature and immature T lymphocytes and a subpopulation of B lymphocytes

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In mammals, the binding of peanut agglutinin (PNA) on the plasma membrane defines subpopulations among lymphocytes from peripheral blood and lymphoid organs. PNA binds Galβ1,3GalNAc residues provided that they are not sialylated. Here, we studied the expression of PNA-binding glycans on healthy horse peripheral blood, thymus, lymph node and spleen lymphocytes. We first demonstrated the binding specificity of PNA for galactose residues by competition experiments and the inhibitory role of sialic acids in PNA binding by sialidase digestion. Unlike human and murine lymphocytes, all equine lymphocytes were found positive by flow cytometry analysis. Double-staining analyses showed that lymphocytes expressing high levels of PNA-binding glycans (PNA^{high} lymphocytes) were made up of the great majority of CD5$^+$, CD4$^+$ and CD8$^+$ cells, and of 30 and 50% of sIg-bearing lymphocytes in peripheral blood and in lymph nodes or spleen, respectively. Lectin histochemistry suggested that lymph node germinal centres contained PNA^{high} B cells. Contrary to what is found in humans and mice, PNA staining intensity on CD5$^+$, CD4$^+$ and CD8$^+$ cells did not differentiate immature from mature T lymphocytes in the equine thymus. The functional consequences of these differences are discussed.
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Abbreviations: **DAB: diaminobenzidine; FITC: fluorescein isothiocyanate; mAbs: monoclonal antibodies; PBA: PBS-BSA (bovine serum albumin)-sodium azide; PBL: peripheral blood lymphocytes; PBMC: peripheral blood mononuclear cells; PBS: phosphate buffered saline; PE: phycoerythrin; PNA: peanut agglutinin; SA-HRP: streptavidin-peroxidase; sIg: surface immunoglobulin.**

Introduction

Like all the cell membranes, the lymphocyte plasma membrane is composed of a lipid bilayer in which glycolipids, proteins, glycoproteins and proteoglycans are inserted. Epitope determinants of a given molecule or common to several molecules can be detected by monoclonal antibodies (mAbs), which allows the characterisation of lymphocyte subpopulations. Carbohydrate residues can be identified by the binding of particular proteins and glycoproteins called lectins. A galactose-binding lectin extracted from *Arachis hypogea*, peanut agglutinin (PNA), is specific for Galβ1,3GalNAc residues [1,2]. In 1976, Reisner *et al.* [3] showed that PNA could separate immature from mature murine thymocytes by an agglutination technique. This was further confirmed by fluorescence microscopy with labelled PNA in mice [4] and humans [5]. More recently, flow cytometry has allowed to specify that PNA-binding glycans are expressed at low level on CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ (single-positive, mature) mouse thymocytes and splenic T cells but also on CD4$^-$CD8$^-$ (double-negative, immature) thymocytes, and at high level on CD4$^+$CD8$^+$ (double-positive, immature) thymocytes [6]. In sheep, horses, goats and dogs, more than 75% thymocytes were found to express PNA receptors [7–10]. In the peripheral blood of these species as well as in domestic cattle, fluorescence microscopy has revealed the presence of PNA-specific glycans on most T cells, but only on a minority of B cells if any [7–11], whereas no lymphocyte was stained in the human blood [5]. Those results were confirmed by flow cytometry for the human, bovine

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and canine species [10,12,13]. In caprine, murine, human and canine spleen and lymph nodes, various proportions of PNA^+ lymphocytes are found [4,5,8,10]. These cells are located in germinal centres, at least in humans and mice [14-16] but also in sheep [14]. Furthermore, PNA^+ cells have been shown to represent germinal centre B cells in the human tonsil [17].

Given the disparity of results obtained from different species, we tried to give the most complete possible picture of PNA-binding glycan expression on the plasma membrane of lymphocyte subpopulations of horse blood and lymphoid organs.

Material and methods

Horses

Horses (8 males, 3 females, aged 2–16 years) from a local riding school or from the Lyon Veterinary School were used for blood sampling. Only animals without infectious diseases, corticosteroid treatment or haematological abnormalities were used, as assessed by clinical examination and leukocyte counts.

Macroscopically normal thymuses (from 2 horses of less than 2 years of age), lymph nodes (one tracheo-bronchial lymph node, one liver hilum lymph node and one mediastinal lymph node) and spleens (2) were collected from 7 different horses in the Lyon-Corbas (France) slaughterhouse.

Preparation of peripheral blood, thymus, lymph node and spleen lymphocytes

For the isolation of peripheral blood mononuclear cells (PBMC), venous blood was collected onto acid citrate dextrose in vacutainer tubes (BD, Le Pont-de-Claix, France) and centrifuged on ficoll diatrizoate (Eurobio, Les Ulis, France) at 600 *g* for 30 min. The cells were resuspended in phosphate buffered saline (PBS) and centrifuged twice through PBS (180 *g*, 15 min to remove platelets and 450 *g*, 10 min wash) before resuspension and counting.

Thymus, lymph node and spleen cells were prepared by teasing through sterile mesh, filtration through gauze and washing 2× in RPMI-1640 culture medium (Gibco BRL, Cergy-Pontoise, France) by centrifugation at 300 g for 10 min. Furthermore, the spleen red blood cells were eliminated by centrifugation over ficoll, and by NH_4Cl lysis if necessary.

All operations were performed at 4°C.

Sialidase treatment

PBMC (3×10^6) were washed in RPMI medium at room temperature and resuspended in RPMI containing 5% inactivated fetal calf serum (Hyclone, Perbio Science, Bezons, France). They were then incubated at the density of 10^6/ml with 10 mU/ml sialidase from *Vibrio cholerae* (Sigma, L'Isle d'Abeau Chesnes, France) for 30 min at 37°C in 5% CO_2. Control cells were incubated under the same conditions but without sialidase.

Treated and untreated cells were then washed in RPMI, resuspended in PBS and counted before fluorescent staining.

Lectins

Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) from *Arachis hypogea* (Vector or EY: Biovalley, Conches, France) was used in 5 μl at 1 mg/ml (lectins from both producers gave similar results) for flow cytometry staining. Biotin-labelled PNA (Vector) was used for histochemistry.

Primary antibodies

Equine surface Ig-bearing lymphocytes were identified using 100 μl of FITC- or biotin-labelled goat Ig $F(ab')_2$ fragments specific for horse Ig $F(ab')_2$ fragments at a 1/2000 dilution (Jackson Immunoresearch, Interchim, Montluçon, France).

The following monoclonal antibodies (mAbs) were used to detect horse CD molecules: CD5 (CVS5, IgG1, kind gift from Dr DP. Lunn, University of Madison, Wisconsin); CD4, clone HB61A, IgG1; CD8α, clone 73/6.9.1, IgG3; CD8β, clone HT14A, IgG1 (VRMD, Pullman, USA). CVS5 was an hybridoma supernatant used in 40 μl while the mAbs from VMRD were used at 15 μg/ml in 20 μl.

The anti-KLH mAb X-40, of IgG1 isotype (BD) was used as negative control for the anti-horse CD mAbs. For FITC-PNA, negative controls consisted of the FITC-labelled goat anti-mouse Ig antibody (initially used as a secondary antibody, see next paragraph) or the FITC-labelled anti-KLH mAb, which displayed the same fluorescence profiles.

Fluorescent staining and flow cytometric analysis

Cell suspensions were adjusted to a concentration of 10^7 cells/ml in PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBA). Aliquots of 5×10^5 cells were incubated with a primary reagent (antibody or PNA) at 4°C in the dark for 30 min, then washed three times in cold PBA. The cells treated only with fluorochrome-labelled reagents were then fixed in 1% formaldehyde in PBA, and those treated with unconjugated or biotinylated antibodies were incubated with secondary reagents before washing (2×) and fixation as above. Unconjugated antibodies were detected with 400 ng of $F(ab')_2$ fragments of goat anti-mouse IgG showing minimum cross-reaction to horse serum proteins and conjugated to FITC (Sigma) or PE (phycoerythrin; Jackson Immunoresearch). The biotin-labelled anti-horse Ig was detected using PE-conjugated streptavidin (BD) at a 1/100 dilution. Incubations with secondary reagents were performed in 20 μl.

In order to determine the specificity of PNA on horse lymphocytes, galactose, glucose or mannose (Sigma) was added to FITC-PNA in some of the assays at a final concentration of 50 or 200 mM, as indicated in results.

Samples for two-colour fluorescence analysis were simultaneously treated with FITC-PNA and the biotinylated anti-horse Ig antibody or the anti-horse CD mAbs (revealed with the PE-labelled anti-mouse antibody).

After labelling, the cells were analysed on a FACScan cytometer using Lysis II software (Becton Dickinson). Peripheral

blood lymphocytes were identified according to their forward scatter and side scatter parameters: lymphocytes have smaller size and granularity than monocytes [18]; this was confirmed by back-gating with the anti-CD5 and sIg Abs. Data from 15,000 events from the global population were recorded for each reagent. The positivity threshold was defined so as that $\leq 1\%$ control cells were positive ('unspecific binding'). The percentage of lymphocytes positive for reaction with each specific mAb or lectin was the difference between the percentage of cells above the threshold and that of unspecific binding.

Lectin histochemistry

Blocks of tissues were embedded in OCT medium, snap-frozen in liquid nitrogen-cold isopentane and stored at –70°C until use. Eight μm-thick cryostat sections were air-dried for 1 h at room temperature and, in some cases, fixed in acetone for 10 min at –20°C. After rehydration in PBS, the sections were incubated with Tissue Conditioner^{TM}from the immunoperoxydase staining Autoprobe II^{TM}kit (Microm, Dardilly, France) for 10 min at room temperature. The slides were then incubated with 10 μg/ml biotinylated PNA for 1 h, washed by two PBS bathes of 5 min and incubated with streptavidin-peroxydase (SA-HRP) for 30 min. After washing, the staining was revealed using diaminobenzidine (DAB) as chromogen; SA-HRP and DAB were taken from the Autoprobe II^{TM}kit. The sections were counterstained with hematoxylin, dehydrated and mounted. Control sections were treated the same way except that PNA was replaced by PBS.

Results

Expression of PNA-binding glycans on horse peripheral blood mononuclear cells

Incubation of cells with PNA did not change size and granularity profiles (data not shown). In every sample, FITC-PNA stained more than 95% of peripheral blood lymphocytes and monocytes. In the present study, when it is written that PNA-binding glycans or PNA ligands are expressed, it is only meant that they are detectable with the technique used, i.e. others may be present but undetectable because they may be hidden by other carbohydrate residues. While monocyte gate cells were stained in a monomodal way, FITC-PNA revealed two populations expressing different levels of membrane PNA-binding glycans, in the lymphocyte gate.

Binding specificity of PNA on horse lymphocytes

The bimodality of PNA ligand expression on PBL incited us to focus on this cell type. The binding specificity of PNA for galactose residues was demonstrated by competition experiments: staining was inhibited by galactose but not by glucose or mannose, in a dose-dependant manner (Figure 1). Staining was globally increased after sialidase digestion (Figure 2).

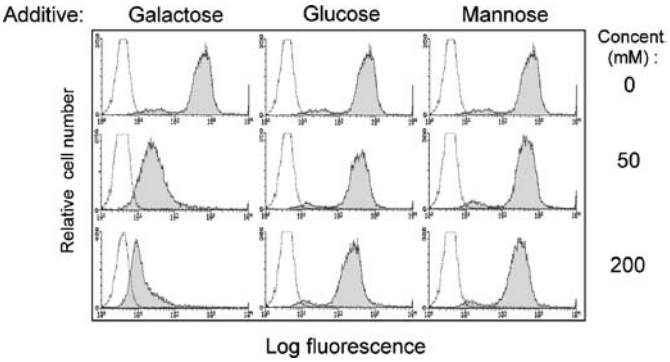

Figure 1. Flow cytometric analysis of the staining of horse PBL by FITC-PNA in the presence of the indicated concentration of monosaccharide. Dotted lines represent labelling by an FITC-labelled goat anti-mouse Ig antibody.

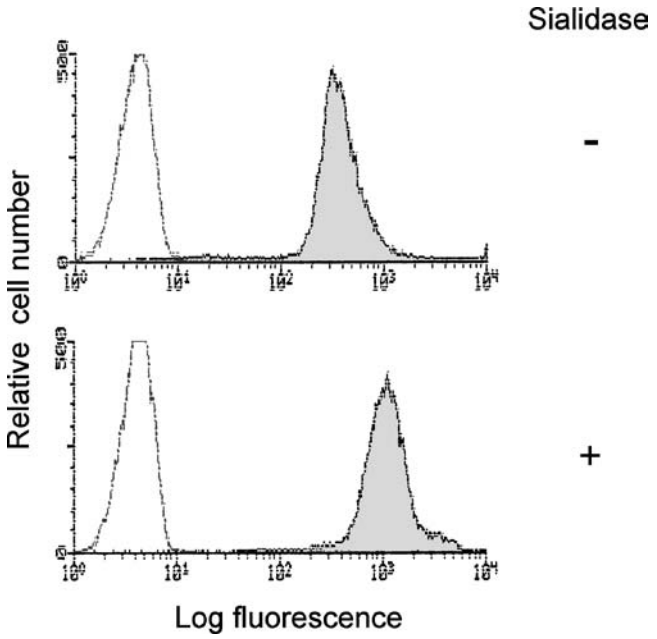

Figure 2. Surface expression of PNA-binding glycans by horse PBL treated or not by sialidase. Dotted lines represent labelling by an FITC-labelled goat anti-mouse Ig antibody.

Expression of PNA-binding glycans on horse peripheral blood, thymus, lymph node and spleen lymphocytes

In every horse examined, more than 95% PBL expressed PNA-binding glycans on their plasma membrane (in means $\pm$ standard deviation: 98.0 $\pm$ 1.0). Similar percentages were found in two thymuses (98.5 and 99%), three lymph nodes (98.6 $\pm$ 0.5%) and two spleens (96 and 96.8%).

However, the expression profiles of PNA ligands differed according to the organs. PBL were distributed between a smaller peak of lower mean fluorescence (PNA^{low} cells, 18.6 $\pm$ 5.9%; $n = 11$ horses) and an extended, greater peak of higher fluorescence (PNA^{high} cells, 79.4 $\pm$ 6.5%). The limit between the two

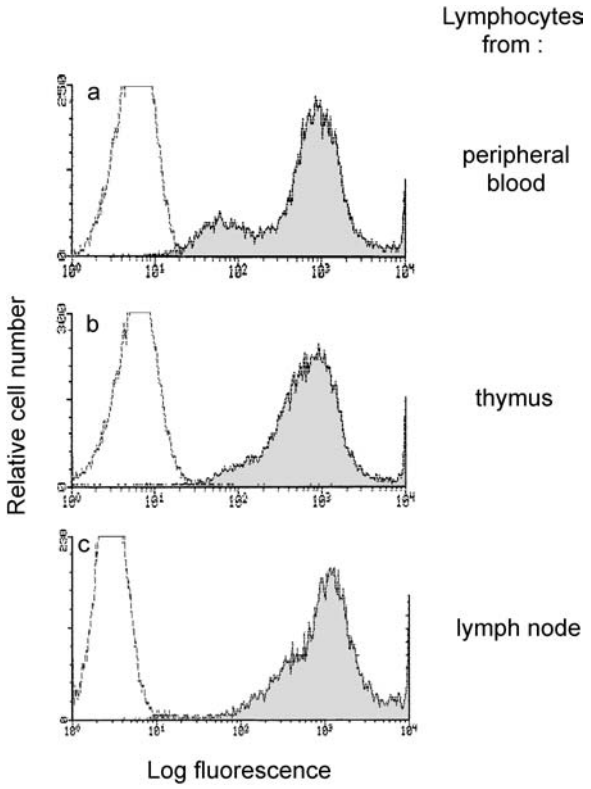

Figure 3. Surface expression of PNA-binding glycans by horse lymphocytes from peripheral blood (a), thymus (b) or lymph node (c). Dotted lines represent labelling by an FITC-labelled goat anti-mouse Ig antibody (a, b) or by the mouse anti-KLH IgG1 X-40 (c).

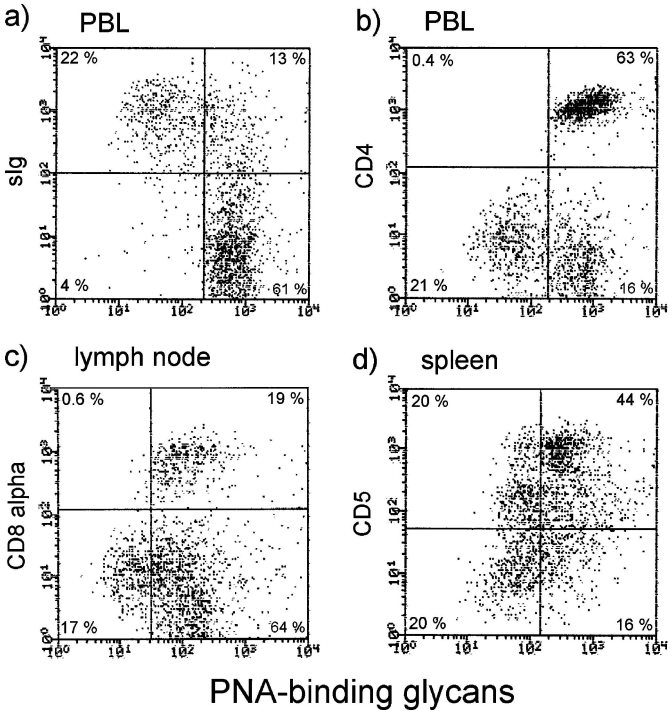

Figure 4. Flow cytometry analysis of horse PBL (a and b), lymph node cells (c) or spleen lymphocytes (d) after double-staining by FITC-labelled PNA and an anti-horse Ig antibody (a), or mAbs directed against horse CD4 (b), CD8α (c) or CD5 (d).

peaks was defined as the middle of the transition zone, which corresponded to cells of intermediate fluorescence (Figure 3a).

In the thymuses, a bimodal distribution was also found but the first peak was shifted to the right and the second one side limits were shifted to the left, making the intermediate population merge into them (Figure 3b). Moreover, the percentage of PNA^{low} cells (8.5–9% in 2 thymuses) was lower than in PBL of most horses.

The cytometry profile given by PNA-FITC on lymphocytes from two lymph nodes looked like that of PBL, with a more progressive transition from PNA^{low} to PNA^{high} cells (Figure 3c). In the 3rd lymph node, fluorescence was less heterogeneous, so that populations could hardly be distinguished. Histograms from the spleens had either one or the other appearance.

Expression of PNA-binding glycans on lymphocyte subpopulations

To know if there was any correlation between the intensity of expression of PNA-binding glycans and the cell line to which lymphocytes belonged, we performed flow cytometry double-stainings with FITC-PNA and anti-horse Ig or CD marker antibodies revealed by PE-labelled reagents.

As shown on Figure 4a, sIg (surface immunoglobulin)-expressing lymphocytes from equine peripheral blood generally expressed low levels of PNA-binding glycans whereas most sIg^- lymphocytes expressed high levels. Indeed, in 6 horses, less than 36% of sIg^+ PBL expressed PNA ligands at higher intensity (means: $30.2 \pm 8.1\%$; Table 1) whereas $93.8 \pm 2.9\%$ of sIg^- cells belonged to the PNA^{high} population.

In lymphoid organs, the high percentage of PNA^{low} sIg^+ cells was less frequent: PNA^{high} cells formed 74% of sIg^+ lymphocytes in 1/2 thymuses, 39 to 61% in 3 lymph nodes (means: $46.2 \pm 12.2\%$) and 47 to 54% in the 2 spleens (Table 1).

The results of double-stainings with anti-horse T cell marker mAbs are illustrated on Figure 4b–d and displayed in Table 1: $98.6 \pm 0.9\%$ of $CD4^+$ PBL (Figure 4b) and $97.4 \pm 2.8\%$ of $CD8\alpha^+$ PBL were PNA^{high} ($n = 4$ horses examined for both markers). These percentages never went below 93 (data not shown).

The PNA^{high} character of $CD4^+$ and $CD8^+$ PBL could also be observed in thymuses, lymph nodes (Figure 4c) and spleens. However, the spleens appeared more heterogeneous, but PNA^{high} cells always made up more than 80% of $CD4^+$ or $CD8^+$ splenocytes.

Some of the samples were double-stained with PNA-FITC and CD8β or CD5 (Figure 4d and Table 1). CD8β always gave results very similar to CD8α. The expression of PNA-binding glycans was linked to a lesser extent to the $CD5^+$ (Figure 4d)

Table 1. Percentages of horse peripheral blood, thymus, lymph node and spleen lymphocyte subpopulations (rows 1, 3, 5, 7 and 9) and of cells expressing high amounts of PNA specific-glycans ('PNA^{high}', rows 2, 4, 6, 8 and 10) among these subpopulations, as determined by flow cytometry. Results are expressed in means ± standard deviation when they were obtained from at least three samples; the number is then written in brackets. Otherwise, the two values are mentioned

% *Lymphocytes*	*Peripheral blood*	*Thymus*	*Lymph node*	*Spleen*
sIg^+	27.0 ± 7.5 (6)	12.4; 5.4	39.6 ± 10.8 (3)	29.5; 30.1
PNA^{high} / sIg^+	30.2 ± 8.1 (6)	32.9; 73.8	46.2 ± 12.2 (3)	53.4; 47.8
$CD4^+$	54.2 ± 8.0 (4)	64.9; 72.2	46.8 ± 7.2 (3)	19.7; 23.5
PNA^{high} / $CD4^+$	98.6 ± 0.9 (4)	92.3; 96.7	95.6 ± 3.7 (3)	97.6; 84.9
$CD8\alpha^+$	17.9 ± 3.3 (4)	53.6; 67.4	15.7 ± 4.0 (3)	19.6; 21.0
PNA^{high} / $CD8\alpha^+$	97.4 ± 2.8 (4)	94.5; 97.9	95.3 ± 3.5 (3)	92.3; 85.0
$CD8\beta^+$	20.9	63.6	11.0; 18.9	21.1; 16.4
PNA^{high} / $CD8\beta^+$	99.2	98.0	96.7; 94.7	90.0; 80.6
$CD5^+$	77.6; 71.8	90.2	56.0; 80.7	61.5; 65.8
PNA^{high} / $CD5^+$	99.2; 89.4	86.2	69.3; 86.2	87.3; 68.7

phenotype: in 5/7 samples, the percentage of PNA^{high} cells among $CD5^+$ lymphocytes was 5–30% lower than that among $CD4^+$ and $CD8^+$ cells (Table 1).

Tissue distribution of PNA-binding glycans in horse lymph node

Labelling of lymph nodes frozen sections was carried out by a peroxydase technique with biotin-labelled PNA. All the lymph node section surface was labelled but germinal centres were more strongly labelled, as well as capillary vessels (Figure 5b–d) and structures of dendritic aspect surrounding follicles (Figure 5d). The labelling was the same on fixed and on unfixed sections.

Discussion

More than twenty years ago, PNA binding was shown to separate horse peripheral blood T lymphocytes from B

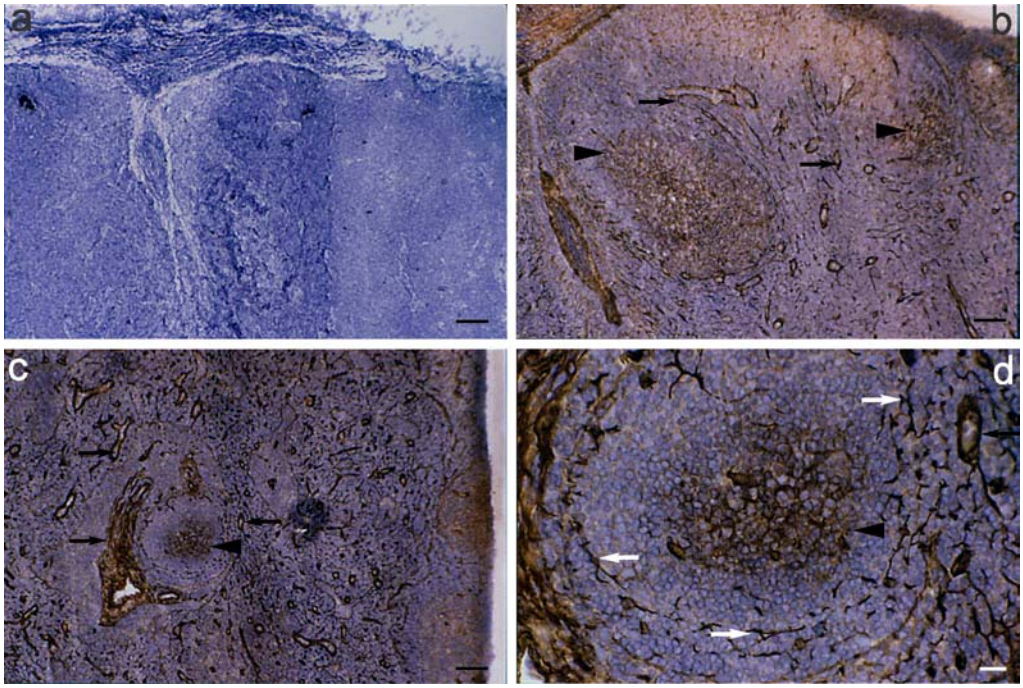

Figure 5. Peroxydase staining of cryostat sections of a horse lymph node with PNA. (a) Control section. (b and c) PNA labelled section, showing germinal centres (thick black arrows) and capillary vessels (thin black arrows); the section zone on b is the same as on a. d) Higher magnification of the follicle shown on c, showing the same type of structures as well as surrounding dendritic structures (white arrows). Scale bars: a, b and c (black bar): 100 μm, d (white bar): 20 μm.

lymphocytes [7]. Our data partly confirm this result. Indeed, whereas T and B cells labelled by FITC-PNA at 0.5 μg/10^6 cells and analysed by fluorescence microscopy are defined as PNA-positive and negative, respectively [7], these cells labelled by 10 μg FITC-PNA/10^6 cells were determined as strongly and weakly positive by flow cytometry in our study (Figure 3). The lectin concentration and the technique sensitivity can explain this difference. Flow cytometry also revealed that 1/3 of peripheral blood B lymphocytes expressed high levels of PNA-binding glycans in our results (Figure 4a and Table 1).

Glycans are linked to the plasma membrane by lipids or amino-acids. We found no difference between the PNA labelling of unfixed and acetone fixed sections. This suggests that the majority of PNA-ligands are not covalently bound to lipids, as it had been shown for human lymphocytes [19].

It has been demonstrated that the absence or the weakness of PNA binding is due to the masking of galactose residues by sialic acids [3,6,20–25]. Our results showed that sialidase digestion increased PNA labelling (Figure 2), which strongly suggests that sialic acids decrease the accessibility of PNA to galactose on horse cells too.

In horse peripheral blood, thymus, lymph nodes and spleen, the great majority of T lymphocytes were PNA^{high} cells, whereas only 30–50% sIg^+ cells were of this phenotype (Table 1).

Ninety-two to 98% of $CD4^+$ or $CD8^+$ thymus cells were PNA^{high} (Table 1), which suggests that most single-positive thymocytes had this phenotype. This contrasts with the human and the murine thymus, in which only immature thymocytes are strongly bound by PNA [4–6]. In mice in particular, $CD4^+CD8^+$ thymocytes are PNA^{high}, while $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ cells are PNA^{low} [6]. That degree of masking of PNA-binding glycans has been proposed to control the egress of thymus cells from the cortex to the medulla [23] or to regulate signal transduction during thymic selection [21]. However, studies on knock-out mice have shown that neither ST3Gal-I (a specific α2,3 sialyltransferase) nor macrophage galactose- and N-acetylgalactosamine-specific C-type lectin (mMGL) appear to play an essential role on T-cell development [26,27]. These data and our results don't necessarily mean that PNA binding glycans represent a meaningless sign of T cell development. However, other sialyltransferase(s) and Galβ1,3GalNAc ligand(s) remain to be identified to explain the role of Galβ1,3GalNAc residues in thymocyte differentiation.

In the blood and the lymphoid organ samples we studied, most T lymphocytes and more than 15% B lymphocytes were PNA^{high}. In humans and mice, resting lymphocytes are either unbound or weakly bound by PNA [4,5,20,22,24,25,28]. The exposure of PNA binding glycans on their T cell surface is increased by the loss of sialic acids upon activation, in vitro [20,22,24,25] and in vivo [28]. In their secondary lymphoid organs, only germinal centre B cells are bound by PNA [14–17]. CD43 deficient mice, which effector and memory $CD8^+$ T cells bound PNA less strongly than similar cell populations from wild-type mice, show a delay in the down-regulation of the immune response and an increased morbidity after chronic viral infection [29]. Besides, ST3Gal-I deficient mice, which T cells are all PNA^+, show a loss of unstimulated $CD8^+$ T cells in the peripheral lymphoid organs via apoptosis [27]. Altogether, these results show the importance of the exposure of PNA-binding glycans in the regulation of the immune response, but also in the homeostatic maintenance of $CD8^+$ T cells.

The striking difference between the results we present here on horses and the situation in humans and mice might imply that Galβ1,3GalNAc residues don't play the same role in lymphocyte activation in the equine and in the human or murine species. However, our results showed that desialylation increased the PNA labelling of horse lymphocytes (Figure 2), which strongly suggests that sialic acids also masked PNA-binding glycans on equine lymphocytes. Furthermore, PNA^{high} sIg^+ B cells were more numerous in lymph nodes and spleens than in peripheral blood. PNA staining on lymph node sections suggests that these cells are located in germinal centres (Figure 5), and are therefore in some state of activation. This could mean that the expression of PNA binding glycans is globally higher in horses than in humans and mice. Moreover, murine CD43 expression affects PNA binding by effector and memory $CD8^+$ T cells, but not by naive $CD8^+$ T cells [29]. Thus, the difference of exposure of PNA binding sites between naive cells and activated or memory cells could be more important than their basic exposure level for the regulation of lymphocyte activation.

Most $CD4^+$ and $CD8^+$ lymphocytes from horse peripheral blood, lymph nodes and spleen expressed high levels of PNA receptors. In most samples on which CD5 labelling was carried out, the frequency of PNA^{high} cells was higher among $CD4^+$ and $CD8^+$ cells than among $CD5^+$ cells. This suggests that some PNA^{low} cells expressed CD5 but not CD4 or CD8. The presence of this cell type had already been suggested by the comparison of single-staining percentages [30]. These cells could be $CD5^+$ B lymphocytes, $CD4^-CD8^-$ T lymphocytes, bearing either the $\alpha\beta$ or the $\gamma\delta$ TCR, or NK lymphocytes. The lymphocyte phenotype of foals suffering from severe combined immunodeficiency suggests that horse NK cells express CD8 but rarely CD5 [31], which nearly excludes the third hypothesis. The existence of $CD5^+$ B lymphocytes in horses is still controversial [30,32–35]. CD3/CD4/CD8 triple-staining or at least CD3/PNA or CD5/sIg double-staining would be necessary to determine which lymphocyte type $CD5^+CD4^-CD8^-PNA^{low}$ cells belong to, but the lack of (commercially) available reagents did not allow us to perform these experiments.

According to our results, the high expression of PNA receptors is not specific to T cells from horse lymphoid organs or even peripheral blood. However, PNA may turn out to be useful in the study of T, B or NK cell subpopulations by double-staining or after fractionation.

Finally, since variations of exposure of PNA-specific glycans are of importance for lymphocyte activation, PNA should

be relevant to the understanding of cell function mechanisms in horses, and studies on horses could contribute towards understanding the role of carbohydrates in the regulation of mammalian cell functions.

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