Identification of glycoproteins that are receptors for peanut agglutinin on immature (cortical) mouse thymocytes

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Received 18 October 1985

Binding of peanut agglutinin is being widely used as a marker for immature mouse thymocytes and for the separation of these cells from the mature thymocytes. Two cell surface glycoproteins that bind peanut agglutinin were detected on unfractionated as well as immature thymocytes by lectin overlay and affinity chromatography: one of M_r between 170000 and 180000, and the other, a minor component, of M_r 110000, both of which are partially sialylated. No receptors for peanut agglutinin were detected on the mature cells, whereas desialylation experiments revealed the presence of a glycoprotein of M_r 110000. These findings were corroborated by electrophoretic analysis of cell surface glycoproteins of the isolated thymocyte subpopulations labeled in their carbohydrate moieties.

Blot analysis *Glycoprotein* *Peanut agglutinin* *Receptor* *Thymocyte subpopulation*

1. INTRODUCTION

Mouse thymus contains two distinct lymphocyte subpopulations: a major one, comprising 85–90% of the total thymocytes, which is immunologically immature and resides in the thymic cortex, and a minor one (10–15%) of immunologically mature cells that resides in the medulla. Earlier work from our laboratory has shown that the immature cells bind peanut agglutinin (PNA), i.e. they are PNA^+, whereas the mature cells do not bind this lectin (PNA^-); the latter cells become PNA^+ upon treatment with sialidase suggesting that the PNA receptors on the mature cells are cryptic [1,2]. Based on the difference in PNA binding between the immature and mature cells, a method was developed, now in wide use, for the separation of these thymocyte subpopulations [1,3]. In this paper we provide information on the molecular characteristics of the PNA receptors on the immature thymocytes and their possible relation to cryptic PNA receptors on the mature cells.

Dedicated to Professor S. Prakash Datta

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNA, peanut agglutinin; PNAr-*n*, receptor for PNA with M_r of $n \times 10^{-3}$

2. MATERIALS AND METHODS

2.1. *Lectins*

PNA was purified by affinity chromatography [4], and was labeled with $Na^{125}I$ using the chloramine T method [5].

2.2. *Cells*

Mouse thymocytes were obtained from male Balb/c mice (6–8 weeks old). They were separated into PNA^+ and PNA^- thymocytes by selective agglutination with PNA [3].

2.3. *Cell surface labeling*

Cells were surface labeled using the lactoperoxidase-$Na^{125}I$ method [6], or the galactose oxidase-NaB^3H_4 method, with or without sialidase pretreatment [7]. The sialic acid moieties

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of glycoconjugates were labeled with NaB^3H_4 after oxidation with $NaIO_4$ [8].

2.4. *Isolation of plasma membranes*

For the isolation of plasma membranes, cells ($0.5–1.0 \times 10^{10}$) were disrupted and homogenized as described [9]. The homogenate (10 ml) was overlaid onto a 42% (w/v) sucrose cushion and centrifuged for 1 h at 100000 × *g* (Beckman SW-27 rotor). The fraction that sediments in the sucrose/buffer interface was collected, diluted 5-fold with 10 mM Tris-HCl (pH 7.2), 150 mM NaCl and 5 mM EDTA, and centrifuged at 100000 × *g* for 1 h (Beckman 60Ti rotor). The pellet was resuspended in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl. All the above steps were done in the cold. Enzymatic markers (5′-nucleotidase and/or alkaline phosphatase) showed that this fraction was at least 12-fold purified, with a yield of about 50%.

2.5. *Cell lysates*

Thymocytes (5×10^8 cells/ml) were lysed in 50 mM Tris-HCl (pH 8.1), 110 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride, 0.5% (v/v) Nonidet P-40, for 30 min at 0°C. Nuclei and cell debris were removed by centrifugation in a Beckman microfuge for 2 min. The supernatant was kept at −20°C until used.

2.6. *Polyacrylamide gel electrophoresis*

Samples were boiled in electrophoresis sample buffer containing 5% (v/v) β-mercaptoethanol for 2 min, and subjected to PAGE in the presence of 0.1% SDS using the Laemmli buffer system [10]. Gels were prepared for fluorography by incubation with Amplify (Amersham Radiochemical Center) according to the manufacturer's instructions.

2.7. *Protein blotting and lectin overlay*

The proteins separated by SDS-PAGE were blotted (2 h) onto nitrocellulose filters in a gradient electric field (40–5 V) [11]. The filters were later quenched in 1% hemoglobin-PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) for 2–16 h at 25°C, incubated in 10 ml of 1% hemoglobin-PBS containing ^{125}I-labeled PNA ($0.5–2.0 \times 10^6$ cpm/ml) for 2 h at 25°C. The filters were then washed with 0.1% Tween 20-PBS for 3 h with changes of buffer every 30 min, and autoradiographed using Curix RP2 Agfa X-ray films for 3–24 h. When indicated, filters were treated with sialidase (0.005 U/ml) for 2–3 h at 37°C in 0.1% hemoglobin-PBS before lectin overlays. Filters were stained for sialic acid using the alkaline phosphatase-hydrazide reagent [12].

2.8. *Lectin affinity chromatography*

PNA (10 mg/g dry beads) was coupled to activated Sepharose 4B (Pharmacia) as recommended by the manufacturer. Plasma membranes solubilized with 1% Nonidet P-40, as well as cell lysates from surface-labeled cells, were centrifuged at 100000 × *g* for 1 h (Beckman 65 rotor) and the supernatant (1–2 ml) was loaded on a PNA-Sepharose 4B column (0.6 × 6.5 cm) in the cold. The unbound material was removed with column buffer (10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.5% Nonidet P-40) until radioactivity reached background level, and the bound material was eluted with 0.2 M lactose in column buffer. Fractions corresponding to the peaks of radioactivity were concentrated using a Diaflo membrane (PM-10) or precipitated with 5 vols cold acetone and submitted to PAGE analysis.

2.9. *Protein determination*

Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as the standard.

3. RESULTS

3.1. *PNA overlay*

PNA overlay analysis of plasma membrane proteins of mouse thymocytes reveals one major band of M_r between 170000 and 180000 (PNAr-170/180), as well as a minor band of M_r 110000 (PNAr-110) (fig.1a). The intensity of the signal observed on the latter band varied between samples, but was always lower than that obtained on PNAr-170/180. The binding of PNA to both glycoproteins could be inhibited by 0.2 M lactose (not shown). After treatment of the blots in situ with sialidase, binding of the lectin to both receptors was enhanced, but the increase was much more pronounced with PNAr-110 (fig.1b). Pretreatment of the plasma membrane fraction with sialidase prior to electrophoresis resulted in a shift of PNAr-110 to higher M_r of approx.

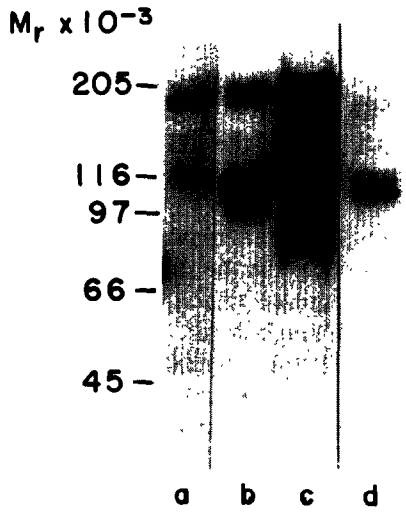

Fig.1. Analysis of the PNA receptors of mouse thymocytes by lectin overlay. Plasma membrane proteins (50 μg) were separated by SDS-PAGE (7.5% acrylamide slab gel), blotted onto nitrocellulose filters and overlaid with ^{125}I-labeled PNA (a,b,c): lane b was treated with sialidase in situ; in lane c the sample was treated with sialidase prior to SDS-PAGE. Sialoglycoproteins were stained on the blot by alkaline phosphatase-hydrazide (d).

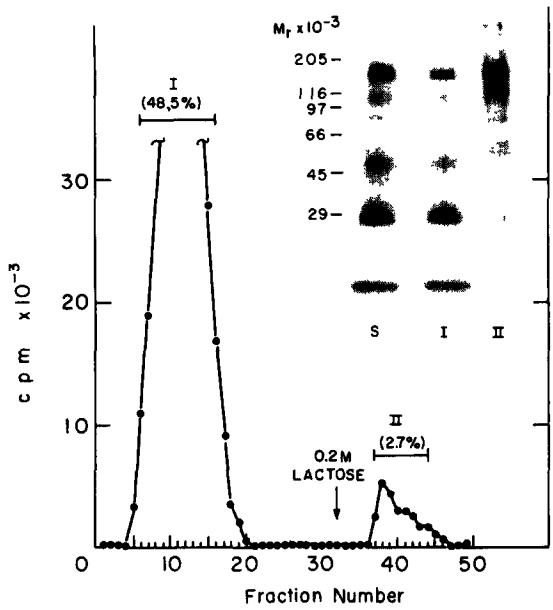

Fig.2. Affinity chromatography on immobilized PNA of plasma membranes of ^{125}I surface-labeled cells. The labeled plasma membranes were solubilized in 1% Nonidet P-40 and applied to a PNA-Sepharose 4B column (0.6 × 6.5 cm). The numbers in parentheses give the recovery of each peak with respect to the material loaded. The peaks of radioactivity were precipitated with acetone and the same amount of counts applied to a 5–15% acrylamide gradient slab gel (inset): S, fraction loaded onto the column; I, fraction unbound; II, fraction eluted with 0.2 M lactose.

135000–145000 (fig.1c). PNAr-110 migrated as the major membrane sialoglycoprotein detected on the blot by staining for sialic acid (fig.1d).

3.2. *Affinity chromatography on immobilized PNA*

Upon affinity chromatography on immobilized PNA of either ^{125}I-labeled plasma membranes (fig.2) or cell lysates, about 2.7% of the material applied was bound specifically to the lectin column. When lysates of cells labeled by galactose oxidase-NaB^{3}H$_4$ were similarly analysed, 2.1% of the radioactivity loaded was bound specifically. Analysis of the material eluted with lactose (fig.2, inset) showed in all the cases that PNAr-170/180 was the major PNA binding component.

3.3. *PNA receptors on immature and mature thymocytes*

Lectin overlay of blots containing the glycoprotein components from immature thymocytes gave a similar pattern to that obtained from plasma membranes of unfractionated cells (fig.3). No binding of PNA was detected on blots of samples derived from mature cells. After in situ treatment of the latter blot with sialidase, PNA binding was only observed to PNAr-110 (fig.3).

The above findings were corroborated by experiments in which the cell surface carbohydrates of the isolated thymocyte subpopulations (PNA$^+$ and PNA$^-$) were labeled prior to PAGE (fig.4). Thus, PNA$^+$ cells labeled by galactose oxidase-NaB^{3}H$_4$ showed a strong band of M_r 180000 which was not detected on PNA$^-$ cells even after pretreatment of the cells with sialidase. This band seems to correspond to PNAr-170/180 detected by PNA overlay. An additional band of M_r 145000 was observed in both subpopulations by labeling of the cells with galactose oxidase-NaB^{3}H$_4$ after treatment with sialidase. The latter glycoprotein, that probably corresponds to the asialo form of

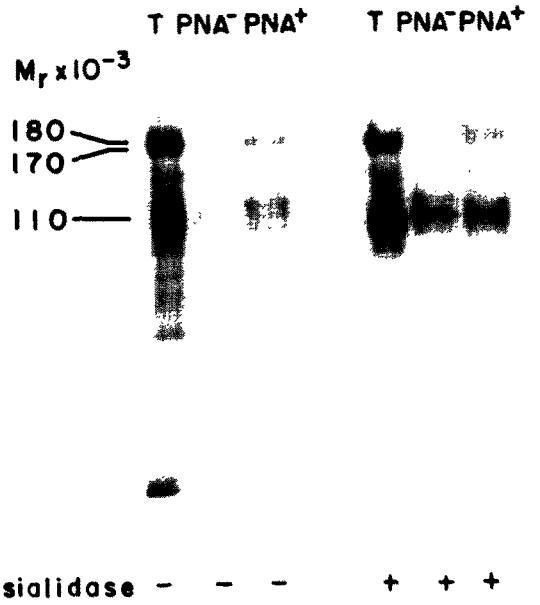

Fig.3. PNA overlay of the proteins of immature and mature thymocyte subpopulations. Plasma membranes of unfractionated thymocytes (T), and lysates of PNA^+ and PNA^- cells (50 μg protein each) were separated by SDS-PAGE (7.5% acrylamide slab gel), blotted onto a nitrocellulose filter and probed with ^{125}I-labeled PNA, with and without sialidase pretreatment in situ.

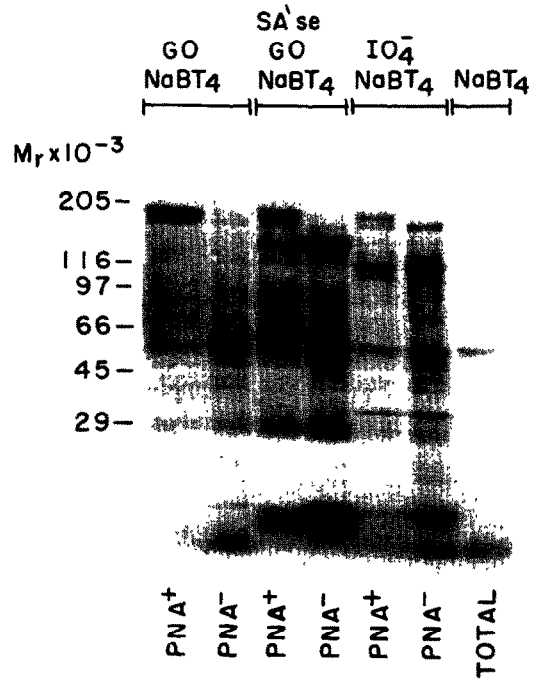

Fig.4. Fluorograms of cell surface labeled thymocyte subpopulations analysed by SDS-PAGE. PNA^+ and PNA^- thymocyte subpopulations separated by agglutination with the lectin were labeled with galactose oxidase-NaB^3H_4 (GO-$NaBT_4$), with galactose oxidase-NaB^3H_4 after pretreatment with sialidase (SA'se-GO-$NaBT_4$), and with $NaIO_4$-NaB^3H_4 (IO_4^--$NaBT_4$). $NaBT_4$ is a control of cells incubated with NaB^3H_4 without any enzymatic treatment. Cells were lysed and the same amount of counts applied onto a 5–15% acrylamide gradient slab gel. The dried gel was exposed for 2.5 months.

PNAr-110, seems to be present in a larger amount on the PNA^- than on the PNA^+ cells. Cells that had been labeled in their surface sialic acid showed a major band of M_r 110000 on both cell subpopulations, although it was more intense on PNA^- cells. In addition 2 bands, of M_r 180000 and 170000, were detected on PNA^+ thymocytes; only the band of M_r 170000 was observed on PNA^- cells.

4. DISCUSSION

Our findings show that the major receptor for PNA on unfractionated as well as on the immature cells corresponds to a glycoprotein of M_r between 170000 and 180000 (PNAr-170/180). This glycoprotein may be related to the family of electrophoretic bands known as T200 of mouse thymocytes [14] and shows characteristics similar to the PNA receptor found by us on human thymocytes (in preparation). A second, minor band of M_r 110000 also binds PNA (PNAr-110), and probably corresponds to the mouse homologue of the leukocyte sialoglycoprotein described on rat thymocytes [15,16].

Data reported by others showed that a glycoprotein similar to PNAr-170/180 was also obtained as the major component by immunoprecipitation of lysates of galactose oxidase-NaB^3H_4 labeled mouse thymocytes using PNA and anti-PNA antibodies [17]. De Petris and Takacs [18] obtained 2 bands, of M_r 100000 and 185000–195000, by PNA affinity chromatography of lysates of cells labeled by the ^{125}I-lactoperoxidase method; however, these authors regarded the latter glycoprotein as a low-affinity receptor since it was hardly detected on

gels or blots by PNA overlay. Brown and Williams [19] could only detect a band of apparent M_r 95 000 by PNA affinity chromatography of lysates of murine thymocytes labeled by $NaIO_4/NaB^3H_4$, perhaps because the PNAr-170/180 was only poorly labeled.

The major difference in PNA binding between the thymocyte subpopulations appears to be due to a lack or decrease in the expression of PNAr-170/180 and to sialylation of PNAr-110. The latter band seems to be present in larger amounts on PNA^- than on PNA^+ cells. The apparent lack of PNAr-170/180 on PNA^- thymocytes suggests that this glycoprotein is not necessary for the immunocompetence of the mature cells, but it may play a role in the interaction of immature thymocytes with the thymic environment.

ACKNOWLEDGEMENTS

This study was supported by the United States-Israel Binational Science Foundation (grant no.2887/82) and the Hermann und Lilly Schilling Stiftung im Stifterverband.

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