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Glucocorticoid agonistic and antagonistic effects of mifepristone and onapristone on thymocyte subset composition and CD26/dipeptidyl peptidase IV activity in infant male rats

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Abstract

Antiglucocorticoid activities of two antigestagens-antiglucocorticoids (AGs)—mifepristone and onapristone—were tested in hydrocortisone-treated suckling male rats. Hydrocortisone (HC) treatment in vivo resulted in (1) reduction of the relative thymus weight and absolute thymocyte counts; (2) relative decrease of the $CD4^+CD8^+$ thymocyte proportion accompanied by an increase of single-positive and double negative thymocyte populations, the latter of which contained large CD3-negative cells expressing a high level of CD26 on their surface; (3) increase of specific dipeptidyl peptidase IV (DPP IV) activity in thymocyte homogenates. Both AGs suppressed the systems (1) and (2) to a comparable extent. When administered alone, mifepristone and onapristone at higher doses exhibited a slight thymolytic effect as revealed by the reduction of the relative thymus weight and thymocyte counts, accompanied by some reduction of the numbers of cycling thymocytes. These effects were limited to the early postnatal period (days 12–17). A comparable agonistic effect of AGs was not observed in systems (2) and (3). Neither HC nor AGs influenced the sialylation pattern of thymocyte membrane bound CD26/DPP IV, which was exclusively of α2,6-type, as demonstrated by analytical isoelectric focusing (IEF) and PAGE analysis in combination with the application of neuraminidases, specific lectins and histochemical staining for DPP IV activity in the gels.

Keywords: Thymocytes; Flow cytometry; Hydrocortisone; Mifepristone; CD26/dipeptidyl peptidase IV; Sialic acid

1. Introduction

The relationship between the suprarenal gland and the thymus was first demonstrated in 1924 by Jaffe [1], who reported growth stimulation of the thymus following total adrenalectomy in young rats. Inomata and Nakamura [2] studied the time course of this phenomenon during the early postnatal development. They did not observe any changes in the weight of the thymus if adrenalectomy in rat pups had been performed before the 16th day of life whereas between postnatal days 21 and 81 the gland excision induced significant hypertrophy of the thymus 5 days later. This indicated that, after weaning, the thymus is under some regulation by the adrenal gland. Circulating steroids, most likely glucocorticoids (GCs), were suspected to be at least partially responsible for the effects observed in rodents. In rats, the level of free corticosterone in plasma rises around the 12th postnatal day and reaches its maximum on day 19, which coincides with the period of weaning [3]. The ability of GCs to reduce the numbers of thymocytes and lymphocytes was documented by Ingle [4] and Dougherty [5]. Later, the underlying mechanism was interpreted as apoptosis [6]. In vivo, approximately 90% of thymocytes die by apoptosis within 48–72 h after steroid treatment [5]. The internucleosomal cleavage of thymocyte DNA is a rapid process beginning already 2 h after GC administration into rats [7]. Small double positive (DP, $CD4^+CD8^+$) cells are the thymocyte population most sensitive to the glucocorticoid-induced cell death [8–10]. The process requires the presence of the GC receptor and is prevented by reagents that block mRNA or protein synthesis in thymocytes [11]. GC-induced thymocyte apoptosis is modulated by the presence of cytokines [12].

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Antigestagens (mifepristone, RU-38.486, and onapristone, ZK 98.299, Schering) display both antiprogestational and antiglucocorticoid effects as has been tested by inhibition of dexamethasone-induced thymolysis in adrenalectomized male rats [13]. In vitro, mifepristone reverted antiproliferative effect of dexamethasone in a murine T-cell line TS1 [14]. The present authors [15] tested early and delayed effects of HC (total dose 75 mg/kg body weight on day 9 and 10) and onapristone on the thymus in suckling male rats. Onapristone in a dose of 50 mg/kg b.w. (body weight) per day injected subcutaneously (s.c.) on days 9–11 inhibited the HC-induced increase of intestinal brush-border enzyme activities on day 12 by 33% and on day 16 by 90%, blocked desialylation of brush-border proteins on day 16, and slightly inhibited thymocyte reduction on day 16. When administered alone, it caused a temporary increase of relative thymus weight by 30–50% on day 12 in two independent experiments and a concomitant increase of DNA/protein ratio. However, no difference between onapristone-treated and untreated animals was found when the thymus weight was tested under the same conditions in another experiment on day 16. These differences in early and delayed effects of HC and onapristone stimulated our detailed investigation of the effect of different doses of HC and of the relative antiglucocorticoid efficiency of different doses of mifepristone and onapristone on thymocyte proliferation and differentiation in terms of thymus weight, absolute thymocyte counts and surface expression of CD4, CD8 and CD26 in infant male rats. Moreover, we were interested, whether AGs would simulate adrenalectomy during the weaning period of rat pups. This hypothesis was not confirmed with the doses of AGs used in our experiments. On the contrary, at the higher doses used in this paper both AGs exhibited a slight agonistic effect during the early postnatal period, as apparent from Section 3.

2. Materials and methods

2.1. Products

Mifepristone (RU-38.486, 17β-hydroxy-11β-[4-dimethylaminophenyl]-17α-propynyl-estra-4,9-diene-3-one) and onapristone (ZK 98.299, 17α-hydroxy-11β-[4-dimethylaminophenyl]-17β-[3-hydroxypropyl]-estra-4,9-diene-3-one) were gifts from Schering AG (Berlin, Germany); hydrocortisone acetate, neuraminidase from *Clostridium perfringens*, papain (*Papaya latex*), 7-aminoactinomycin D (7-AAD), MW-SDS-Blue (prestained Kit for molecular weights), protease inhibitor cocktail, pepstatin A and 4-(2-aminoethyl) benzenesulfonyl fluoride, gelatin and sodium azide were from Sigma (St. Louis, MO, USA); R-PE-conjugated mouse anti-rat CD4 monoclonal antibody (mAb) OX-35, FITC-conjugated mouse anti-rat CD8 mAb OX-8, and R-PE-conjugated mouse anti-rat CD26 mAb OX-61 were products of Pharmingen (San Diego, CA, USA). The unconjugated OX-61 and FITC-conjugated mouse anti-rat CD3 mAb IF4 were purchased from Serotec (Oxford, UK). Biotinylated *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) as well as streptavidin-conjugated peroxidase and alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium chloride and neuraminidase from *Newcastle disease virus* were from Boehringer (Mannheim, Germany). Goat anti-mouse HRP (horse-radish peroxidase) was obtained from Bio-Rad (Hercules, CA, USA). Zwittergent 3–14 was from Calbiochem (La Jolla, CA, USA); glycyl-L-proline-(4-methoxy-2-naphthylamide) was from Bachem Feinchemikalien (Bubendorf, Switzerland); glycyl-L-proline-4-nitroanilide was from Merck (Darmstadt, Germany) and 4-chloro-1-naphthol was purchased from Fluka (Buchs, Switzerland). Fast Blue B salt and Coomassie Brilliant Blue R were from Serva, Feinbiochemica (Heidelberg, Germany); Agarose IEF, Pharmalyte™ 3–10 and Isoelectric Focusing Calibration Kit were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden) and bovine serum albumin from Mann Research Laboratories (New York, NY, USA).

2.2. Animals

Wistar rat male pups were used in all experiments approved by ethical committees of the Institutes involved. The litter size was adjusted to 8–10 male pups that remained with the dams in normal cages until sacrifice.

2.2.1. Treatment with hydrocortisone and antigestagens-antiglucocorticoids (AGs) mifepristone and onapristone

The drugs and/or saline were administered s.c. during the morning hours (9–10 a.m.). Unless otherwise stated, 9-day-old male rats were given a single dose of hydrocortisone acetate in saline, 6 mg/kg b.w. on day 9 and/or (40 mg/kg b.w.) per day AGs in saline on days 9–11. Sham-treated animals were used as controls. Animals were sacrificed on day 12. Some onapristone-treated animals were sacrificed on day 16 to study the effect of onapristone on the relative weight of adrenals. In another set of experiments the pups received both hydrocortisone acetate and AGs in a double dose, i.e. 12 mg/kg b.w. and 3 × 80 mg/kg b.w., respectively.

The effect of AG treatment during postnatal development: Five groups of male rats received single doses of 2 mg per day mifepristone (or onapristone) in saline on three successive days starting at the age of 9, 12, 14, 16 and 23 days, respectively (i.e. (80 mg/kg b.w.) per day on days 9–11 of the youngest group and (28 mg/kg b.w.) per day on days 23–25 of the oldest group). One day after the last administration, i.e. on day 12, 15, 17, 19 and 26, respectively, the animals were sacrificed. Age-matched, sham-treated littermates were used as controls.

2.2.2. Thymocyte preparation

Upon sacrifice, the thymus was excised; its weight was compared with the body weight of the animal and expressed as the relative weight in g/kg b.w. Thymocytes were released by teasing with a pair of forceps followed by repeated pipetting with a polypropylene Pasteur pipette. Tissue debris was sedimented at room temperature and $1 \times g$ for 1 min. The cell suspension was centrifuged three times ($400 \times g$, 10 min, 4 °C) and resuspended in ice cold washing buffer (phosphate-buffered saline, PBS) containing in addition 0.2% gelatin and 0.1% sodium azide (washing and staining buffer, WSB) for flow cytometry measurements. After the last washing step, the cells were counted and their density was set as required for subsequent use.

2.2.3. Thymocyte membrane isolation

Cytoplasmic membranes were isolated by a modified method of Jin et al. [16] using hypotonic lysis. Briefly, washed cells (150–200 millions) were incubated for 15 min on ice in 1 ml of hypotonic buffer (42 mM KCl, 10 mM Hepes, pH 7.4, 5 mM $MgCl_2$) containing a protease inhibitor cocktail as recommended by the manufacturer. The cell suspension was then passed 5 times through a 25 gauge needle in the cold and centrifuged for 10 min at $200 \times g$ and 4 °C to pellet the nuclei. The supernatant was transferred into an Eppendorf tube and the heavy membrane fraction was collected at $10{,}000 \times g$ for 10 min in the cold. The pellet was suspended in 10 mM KCl for further use in polyacrylamide gel electrophoresis (PAGE) or isoelectric focusing (IEF).

2.3. Methods

2.3.1. Flow cytometry

For surface staining, 1×10^6 cells in 100 μl of WSB were incubated in a U-shaped microtiter plate with a cocktail of FITC- and R-PE-conjugated monoclonal antibodies (mAbs) directed against rat leukocyte surface markers and 100 μl of WSB containing 10% heat inactivated non-immune mouse serum to prevent Fc-receptor-mediated binding of immunoreagents. After a 30 min incubation, the cells were washed three times in WSB. Before measuring, the cells were resuspended in 100 μl of WSB and 10 μl of propidium iodide (PI, 10 μg/ml) in PBS was added to stain nucleic acids in damaged and dead cells. DNA content on the single cell level was determined using the DNA intercalating probe 7-AAD as described by Šinkora et al. [17]. Briefly, the cells were washed, centrifuged and fixed with cold (−20 °C) 70% ethanol for 1 h, centrifuged ($2000 \times g$, 10 min, 4 °C) and washed in PBS. The pellet was then incubated with 100 μl of 7-AAD in PBS (40 μg/ml) for at least 20 min at 4 °C in the dark until measurement. Data were acquired on a standard FACSort flow cytometer (Becton Dickinson, BD, San Jose, USA) and analyzed using the PC-LYSYS II software (BD). In DNA content analysis, the Doublet Discrimination Module was used for a single cell event (singlet) discrimination from doublets and higher multiplets. The proportion of cells in the S + M phase was determined in the singlet population by region gating in the two-dimensional histogram of the 7-AAD fluorescence peak area versus 7-AAD fluorescence peak width. In surface staining, the damaged and dead cells were excluded from analysis by gating for PI-negative events.

2.3.2. Analytical isoelectric focusing (IEF)

Analytical IEF of Zwittergent 3–14 (0.05% concentration) and papain [18] solubilized thymocyte membrane fraction was performed on a thin Agarose IEF layer using Pharmalyte with pH intervals 3–10. Proteins in the gel were stained by Coomassie Brilliant Blue R solution. Dipeptidyl peptidase IV activity in the gel was demonstrated by simultaneous azo-coupling reaction with 0.5 mM glycyl-L-proline-(4-methoxy-2-naphthylamide) and Fast Blue B at pH 7.2 as in [19]. For the demonstration of sialic acid bound to dipeptidyl peptidase IV, the solubilized membrane fraction was incubated before IEF separation with neuraminidase from *Clostridium perfringens* as in [19] and compared after IEF separation on the focusogram with the pI of dipeptidyl peptidase IV in the untreated samples.

2.3.3. PAGE analysis

The heavy membrane fraction was solubilized under non-denaturing conditions in non-reducing Laemmli-sample buffer containing 0.1% SDS. Proteins separated by 7.5% SDS-PAGE were electroblotted onto the nitrocellulose membrane and stained with biotinylated SNA or biotinylated MAA, the binding of which was visualized by the streptavidin-alkaline phosphatase complex and 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (X-phosphate/NBT). To remove sialic acid from the membrane sample an overnight incubation with 3 mU of neuraminidase from *C. perfringens* or 6 mU of neuraminidase from *Newcastle disease virus* was performed at 37 °C prior to PAGE run. The glycoproteins fetuin containing α2,3-linked sialic acid residues and transferrin with α2,6-linked sialic acid residues served as positive or negative controls. The unconjugated mouse OX-61 anti-rat CD26 was used to prove the presence of CD26. The binding of the antibody was visualized by goat anti-mouse-HRP (horse-radish peroxidase) complex and 4-chloro-1-naphthol. Dipeptidyl peptidase IV activity in the blot was demonstrated by azo−coupling reaction as in IEF gel.

2.3.4. Enzyme activity of dipeptidyl peptidase IV

Dipeptidyl-peptide hydrolase (EC 3.4.14.5, DPP IV) in thymocyte homogenates was determined with 1.4 mM glycyl-L-proline-4-nitroanilide at pH 8.0 and the released chromogen was measured at 405 nm as in [15].

Protein content in thymocyte homogenates was determined by the method of Lowry et al. [20].

3. Results

3.1. Antigestagens-antiglucocorticoids suppress hydrocortisone effect on thymus weight and thymocyte counts in vivo and exert glucocorticoid-like effects when applied alone

In our previous experiments [15] we administered a dose of 75 mg hydrocortisone acetate/kg b.w. to demonstrate the effect of onapristone on intestinal brush-border enzymes, their sialylation and the relative thymus weight in infant male rats. However, for the demonstration of how HC influences individual thymocyte subsets, this dose appeared to be too high as it resulted in a dramatic drop of total thymocyte counts to less than 1% of that in control animals. Therefore, we have changed the experimental setup and used 6 or 12 mg of HC/kg b.w in our current experiments. For the demonstration of antiglucocorticoid effect of mifepristone and onapristone, the total dose delivered in aliquots during three successive days was 120 or 240 mg/kg b.w.

In one set of experiments, groups of three-to-four 9-day-old male rats were given s.c. 6 mg HC/kg b.w. on day 9 and (40 mg mifepristone/kg b.w.) per day on days 9–11. Other groups were given HC or mifepristone only. The fourth group of sham-treated animals served as a control. All animals were sacrificed on day 12. The relative thymus weight and the thymocyte count were evaluated.

HC decreased both the relative thymus weight and absolute thymocyte numbers (Fig. 1). Mifepristone suppressed the HC effect on the relative thymus weight and reversed its effect on absolute thymocyte counts. However, mifepristone alone slightly decreased the relative weight of the thymus.

In another set of experiments the suckling rats received both HC and AGs in a double dose. A group of three-to-four 9-day-old male rats was given s.c. 12 mg HC/kg b.w. In other two groups, 12 mg HC/kg b.w. on day 9 was combined with (80 mg/kg. b.w.) per day of mifepristone or onapristone on days 9–11. The fourth group was a sham-treated control. All animals were sacrificed on day 12. The relative thymus weight and absolute thymocyte counts are shown in Fig. 2. It is apparent that, at a higher dose, HC decreased the relative thymus weight down to 48%. However, the thymocyte count represented only 10% of that in the controls. AGs suppressed the HC effect on both the relative thymus weight and absolute thymocyte numbers. On the other hand, when administered alone, mifepristone as well as onapristone decreased the relative thymus weight and thymocyte counts, with onapristone being a little bit more effective (Fig. 3).

3.1.1. Effect of antiglucocorticoids on the relative thymus weight, thymocyte number and cycling thymocytes during postnatal development

Since adrenalectomy influenced positively the relative weight of thymus after the 21st postnatal day [2] and since the secretion of corticosterone in plasma rises around the 12th postnatal day reaching its maximum on day 19 [3], we have decided to test the influence of mifepristone during postnatal development in infant male rats. Three-to-four animals in each group were injected with 2 mg per day of mifepristone on 3 successive days starting at the age of 9, 12, 14, 16 and 23 days, respectively. One day after the last injection the animals were sacrificed and both the weight of the thymus and thymocyte counts were compared with the same parameters in age-matched controls. As neonatal rats grow rapidly, the body mass in the control group increased from 27.52 ± 0.35 to 70.42 ± 3.51 g between days 12 and 26. Similarly, in mifepristone-treated animals, a body mass increase from 25.82 ± 0.81 to 71.4 ± 1.78 g was recorded. When the total dose of mifepristone is referred to body mass, the values of 232 and 84 mg/kg b.w. weight are obtained in the youngest and oldest group, respectively. Although the dosage at different age varied significantly, the minimal amount of the active substance (3×28 mg/kg b.w.) was always higher than usual in other clinical and experimental studies (see Section 4). Results are shown in Table 1. Until day 17, mifepristone significantly decreased the relative thymus weight, which was accompanied by a

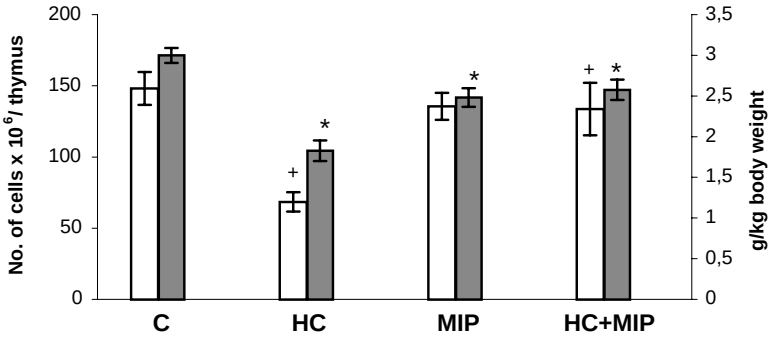

Fig. 1. Effect of HC (hydrocortisone, 6 mg/kg b.w.), MIP (mifepristone 3×40 mg/kg b.w.) and HC + MIP (6 mg + 3×40 mg/kg b.w.) on the relative thymus weight (g/kg b.w., filled bars), and thymocyte counts (number of cells $\times 10^6$, open bars). The values are expressed as means ± S.E.M. (∗) Significant difference in g/kg b.w., $P < 0.0001$ (HC), $P < 0.05$ (MIP), $P < 0.01$ (HC + MIP) vs. controls; $P < 0.01$ (HC + MIP) vs. HC. $^+$ Significant difference in number of cells, $P < 0.001$ (HC) vs. control; $P < 0.05$ (HC + MIP) vs. HC.

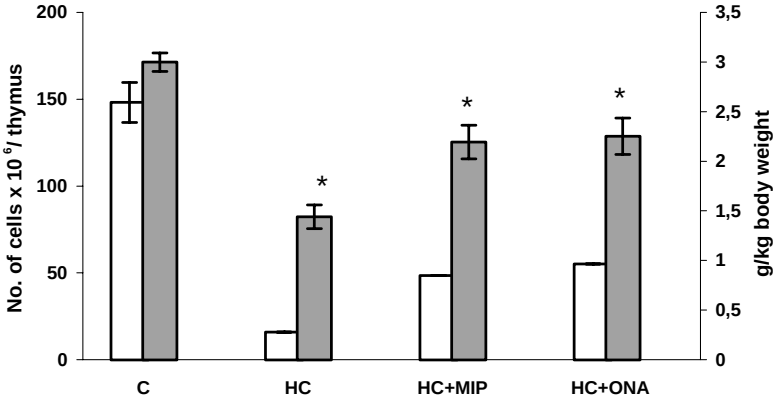

Fig. 2. Effect of HC (hydrocortisone, 12 mg/kg b.w.), HC + MIP (hydrocortisone + mifepristone, 12 mg + 3 × 80 mg/kg b.w.) and HC + ONA (hydrocortisone + onapristone, 12 mg + 3 × 80 mg/kg b.w.) on the relative thymus weight (g/kg b.w., filled bars) and absolute thymocyte counts (number of cells × 10^6, open bars). The values are expressed as means ± S.E.M. Open bars without S.E.M. represent mixed samples obtained from three-to-four thymuses (due to low thymocyte counts per thymus). (∗) Significant difference in g/kg b.w., $P < 10^{-8}$ (HC), $P < 0.001$ (HC + MIP), $P < 0.001$ (HC + ONA) vs. control; $P < 0.01$ (HC + MIP), $P < 0.01$ (HC + ONA) vs. HC.

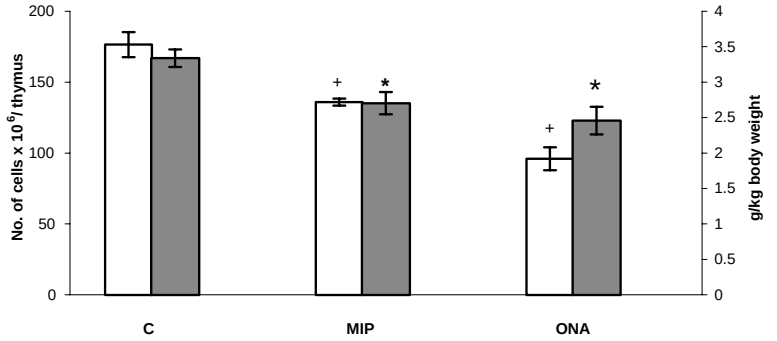

Fig. 3. Effect of MIP (mifepristone, 3 × 80 mg/kg b.w.) and ONA (onapristone, 3 × 80 mg/kg b.w.) on the relative thymus weight (g/kg b.w., filled bars), and absolute thymocyte counts (number of cells × 10^6, open bars). The values are means ± S.E.M. (∗) Significant difference in g/kg b.w., $P < 0.05$ (MIP), $P < 0.01$ (ONA) vs. control. (+) Significant difference in No of cells × 10^6, $P < 0.01$ (MIP), $P < 0.001$ (ONA) vs. control.

drop in the total thymocyte numbers. This confirmed the thymolytic effect of AGs described in Figs. 1 and 3. From these figures it is evident that both AGs decrease the relative thymus weight and thymocyte counts and that the effect is more pronounced at higher doses of AGs. At the dose of 3 × 2 mg, both AGs decreased the relative proportion of cycling cells (S + M phase) in 12-day-old rats (Table 2), whereas an opposite effect was observed in 19-day-old rats. No influence on the specific activity of DPP IV, which did not exceed 0.3 nkat/mg protein, was observed (not shown). At the higher doses of both AGs, no positive effect on thymus growth that would resemble adrenalectomy was observed during the postnatal development of infant male rats.

Table 1
Changes in relative thymus weight (g/kg body weight) and in number of thymocytes (10^6 cells per thymus) during postnatal development

Age (day)	Relative thymus weight		10^6 cells per thymus	
	Control	Mifepristone	Control	Mifepristone
12	3.338 ± 0.123	2.703 ± 0.157[a]	176.5 ± 8.8	135.9 ± 2.5[b]
15	4.317 ± 0.311	3.448 ± 0.094[a]	171	153
17	4.068 ± 0.113	3.519 ± 0.092[b]	219	195
19	3.933 ± 0.212	3.852 ± 0.120	210.1 ± 12.4	222.9 ± 17.1
26	3.487 ± 0.242	3.356 ± 0.126	306	266

Three successive doses of 2 mg per day of mifepristone were administrated 3, 2 and 1 day(s) before sacrifice. The values are expressed as means ± S.E.M. The values without S.E.M. represent mixed samples obtained from four thymuses.

[a] $P < 0.05$.

[b] $P < 0.01$ vs. control.

Table 2
Effect of mifepristone and onapristone on the percentage of cycling thymocytes (S + M phase)

Age (day)	Cycling thymocytes (%)		
	Control	Mifepristone	Onapristone
12	10.30 ± 0.33	7.26 ± 0.25[a]	7.39 ± 0.47[b]
19	8.30 ± 0.15	9.17 ± 0.27[c]	10.40 ± 0.08[a]

Three successive doses of 2 mg per day of mifepristone or onapristone were administrated 3, 2 and 1 day(s) before sacrifice. The values are expressed as means ± S.E.M.
[a] $P < 0.0001$ vs. control.
[b] $P < 0.001$.
[c] $P < 0.05$.

We have considered the possibility that influencing the regulation of hypothalamic–pituitary–adrenal (HPA) axis may be involved in the thymolytic activity of AGs. After (40 mg/kg b.w.) per day of onapristone on postnatal days 9–11, the relative weight of adrenals (mg/kg b.w.) increased significantly by 15% and nonsignificantly by 39% on day 12 and 16, respectively (Table 3).

3.1.2. *Thymocyte subsets characterized by CD4, CD8 and CD26 expression: HC and AG effects*

In rats treated with a lower dose of HC (6 mg/kg b.w.), the decline of thymocyte numbers down to 40–50% (Fig. 1) was accompanied by a relative increase of mature, single-positive (SP, $CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes and double negative (DN, $CD4^-CD8^-$) cells as shown by flow cytometric (FCM) analysis (Fig. 4A). This can be explained by reduction of DP ($CD4^+CD8^+$) cortical thymocytes (compare Fig. 4A and D), which are well-known to be sensitive to GC treatment (see also Fig. 5). Figs. 4B and 5 confirm that mifepristone (3×40 mg/kg b.w. or 3×80 mg/b.w.) suppresses the HC-mediated DP thymocyte depletion. No considerable effect of mifepristone administered alone on CD4/CD8 thymocyte subsets could be detected by FCM analysis (Fig. 4C).

Similarly, after treating suckling rats with a higher dose of HC (12 mg/kg b.w.), the relative proportion of DP thymocytes decreased markedly. As in the case of lower doses, mifepristone and onapristone (3 × 80 mg/kg b.w.) substantially antagonized the HC effect (Fig. 5).

To show whether GC treatment might have any effect on thymocyte DPP IV expression and activity, we have studied CD26 density on the surface of thymocyte subsets and DPP IV activity in total thymocyte lysates in selected

Table 3
Effect of onapristone on relative weight of adrenals (mg/kg b.w.)

Age (day)	Relative weight of adrenals	
	Control	Onapristone
12	470.42 ± 17.22	540.40 ± 29.37[a]
16	411.27 ± 48.87	572.33 ± 54.91

Administration of onapristone: (40 mg/kg b.w.) per day on days 9–11 Values are means ± S.E.M.
[a] $P < 0.05$ vs. control.

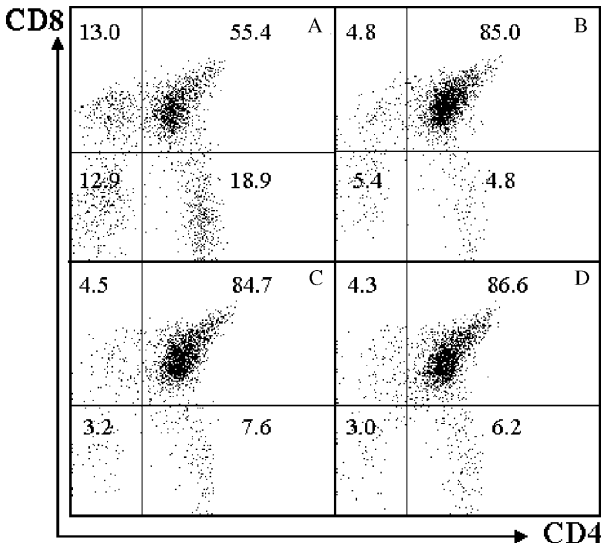

Fig. 4. An example of CD4 and CD8 expression on the surface of thymocytes isolated from 12-day-old rat males treated with 6 mg/kg b.w. of hydrocortisone on day 9 (A), 6 mg/kg b.w. of hydrocortisone on day 9 and three single doses (days 9–11) of (40 mg/kg b.w.) per day of mifepristone (B), mifepristone alone (C) and sham-treated age-matched controls (D). Proportions of double negative (lower left quadrants in CD4/CD8 dot plots), double positive (upper right) and single-positive (lower right for $CD4^+CD8^-$ and upper left for $CD4^-CD8^+$) thymocyte subsets are depicted as numbers representing the percentage of events. A depleting effect of HC on the double positive thymocyte population (compare A and D) and the antagonistic effect of mifepristone to HC (compare A and B) are apparent.

experimental groups. Fig. 6A and B confirm the findings of Bauvois [21] and Bristol et al. [22] that, while all stages of thymocyte differentiation are CD26-positive, the earliest $CD4^-CD8^-$ precursors bear the highest amount of CD26 on their surface (Fig. 6A). These cells are large and the

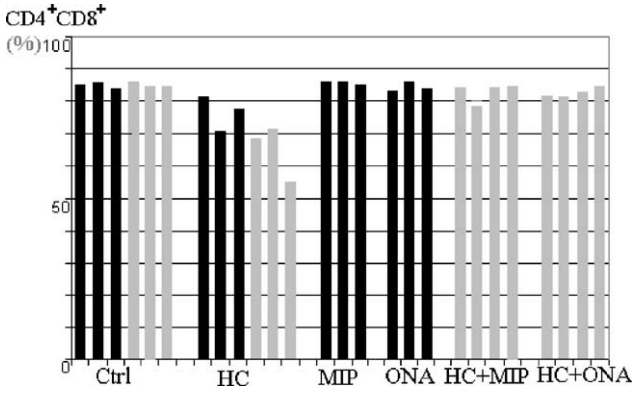

Fig. 5. Diagram showing the proportion of double positive (DP, $CD4^+CD8^+$) cells among thymocytes isolated from 12-day-old male rats treated with 12 mg/kg b.w. of HC and/or three single doses (days 9–11) of (80 mg/kg b.w.) per day of antigestagens. The results are shown from two independent experiments; each bar represents one rat; black bars: experiment No. 1, gray bars: experiment No. 2. HC: hydrocortisone; Ctrl: control; MIP: mifepristone; ONA: onapristone; HC + MIP: hydrocortisone + mifepristone; HC + ONA: hydrocortisone + onapristone. The antagonistic effect of both antigestagens to hydrocortisone is apparent.

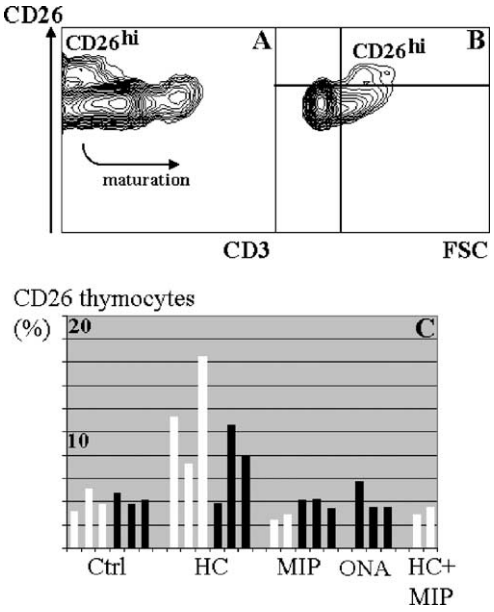

Fig. 6. Surface CD26 expression on thymocytes. Fluorescein isothiocyanate (FITC) anti CD3 and phycoerythrin (PE) anti-CD26 monoclonal antibodies were used. (A) CD3/CD26 counterstaining of thymocytes isolated from sham-treated 12-day-old male rats. CD26-high cells are present among the earliest CD3-negative precursors. These cells are CD4- and CD8-negative (not shown). (B) CD26-high cells are large and their proportion can be easily determined in the FSC/CD26-fluorescence dot plot (upper right quadrant). (C) The percentage of large CD26-high cells among isolated thymocytes in 12-day-old rat pups treated with saline (Ctrl), or hydrocortisone (HC), mifepristone (MIP), onapristone (ONA) and hydrocortisone + mifepristone (HC + MIP). Black bars (three rats): 12 mg HC/kg b.w.; 3 × 80 mg MIP (ONA)/kg b.w.; white bars (two-three rats): 6 mg HC/kg b.w.; 3 × 40 mg MIP/kg b.w.

FSC/CD26-fluorescence dot plot (Fig. 6B) is thus convenient for unambiguous enumeration of CD26-high cells in CD26-single stained thymocyte suspensions. Relative proportions of large CD26-high precursors in different experimental groups are shown in Fig. 6C. It is obvious that glucocorticoid treatment results in a dramatic increase of $CD26^{hi}$ thymocyte proportion and mifepristone antagonizes this effect.

In agreement with FCM findings, HC in a dose of 6 mg/kg b.w. significantly increased the overall DPP IV activity in the total thymocyte population from 0.3 nkat/mg protein to 0.373 nkat/mg protein. Mifepristone at the dose of 3 × 40 mg/kg b.w. did not antagonize the HC-effect in this case, neither had it any effect on DPP IV activity by itself (Fig. 7).

3.1.3. Sialylation of dipeptidyl peptidase IV in thymocyte plasma membranes after HC and AG treatment: IEF and PAGE analysis

Analytical isoelectric focusing of DPP IV in solubilized thymocyte plasma membranes isolated from 12-day-old infant rat males treated with HC (6 mg/kg b.w.) and/or mifepristone or onapristone (3 × 40 mg/kg b.w.) revealed sialylated forms of DPP IV, the presence of which was demonstrated by the shift of pI from 4.5 to 5.5 after *C. perfringens* neuraminidase treatment of solubilized membranes in vitro. Unlike the intestinal DPP IV [15,18], there was no influence of HC or AG on the sialylation pattern of DPP IV in rat thymocytes (Fig. 8).

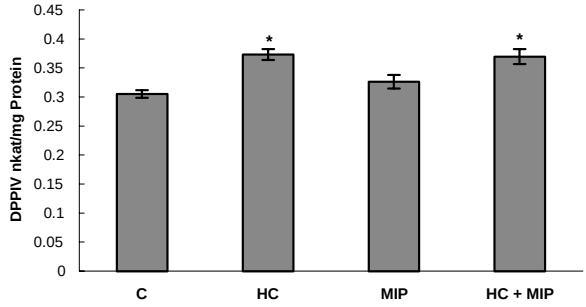

Fig. 7. Overall DPP IV activity in total thymocyte population. Substrate glycyl-L-proline-4-nitroanilide. C, control; HC, hydrocortisone 6 mg/kg b.w.; MIP, mifepristone 3 × 40 mg/kg b.w.; HC + MIP, hydrocortisone (6 mg/kg b.w.) and mifepristone (3 × 40 mg/kg b.w.) treated. Results are means ± S.E.M. of 4–6 litters of 12-day-old rats. (*) Significant increase of DPP IV activity, $P < 0.001$ vs. control.

Fig. 9 demonstrates that sialic acid linkage in the oligosaccharide chains in dimeric [23] CD26/DPP IV is of α2,6-type. CD26/DPP IV run in PAGE under non-reducing non-denaturing conditions retained enzymatic activity as demonstrated by enzyme activity staining (Fig. 9, lanes

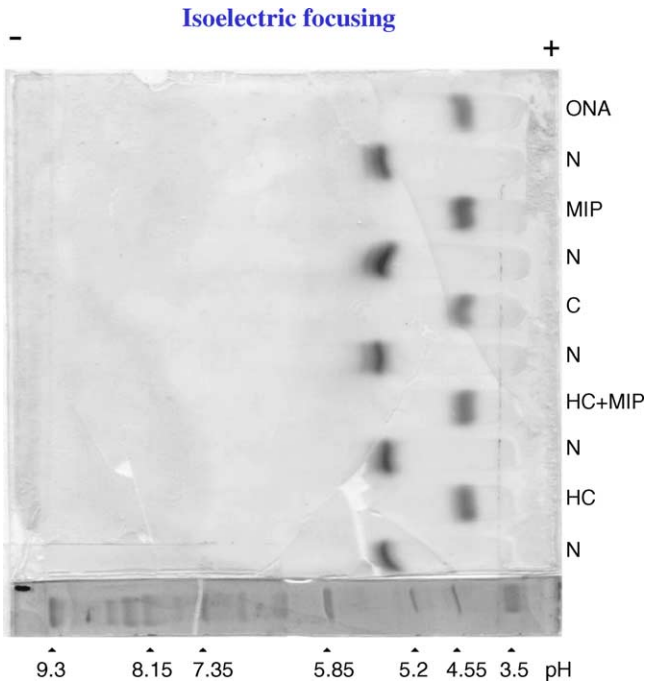

Fig. 8. Analytical isoelectric focusing of dipeptidyl peptidase IV from solubilized thymocyte membranes of 12-day-old rats in thin layer of Agarose IEF (3–10) gel. Staining: substrate Gly-pro-4-methoxy-naphthylamide, Fast Blue B. The zymogram bands were compared with pI values of known proteins in the calibration kit. C, control; HC, hydrocortisone 6 mg/kg b.w.; MIP, mifepristone 3 × 40 mg/kg b.w.; ONA, onapristone 3 × 40 mg/kg b.w.; HC + MIP, hydrocortisone and mifepristone-treated; N, treated with neuraminidase from *C. perfringens*.

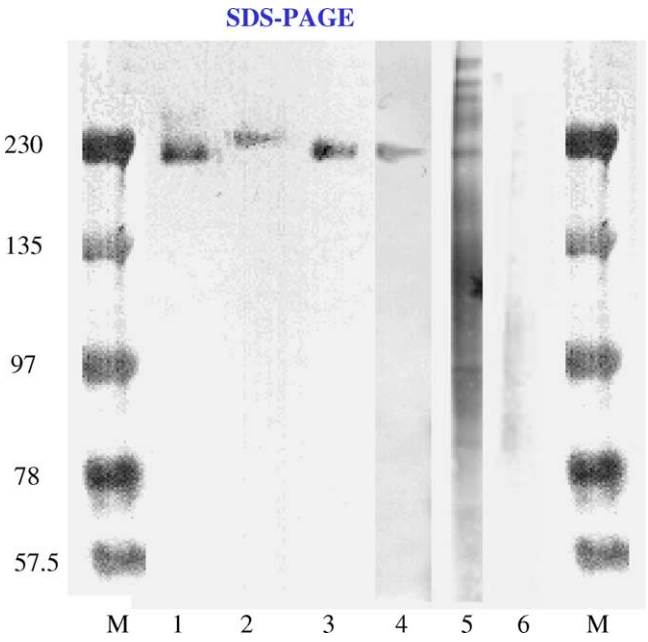

Fig. 9. CD26/DPP IV in a PAGE-separated rat thymocyte membrane preparation. Gels were run under nondenaturing and nonreducing conditions. M, molecular weight markers; 1, DPP IV activity, azo-coupling staining with Gly-L-Pro-(4-methoxy-2-naphthylamide) and Fast Blue B; 2, band of DPP IV activity after treatment with neuraminidase from *C. perfringens*; 3, band of DPP IV activity after treatment with neuraminidase from *Newcastle disease virus*; 4, staining with CD26 mouse monoclonal antibody, goat anti-mouse second antibody conjugated peroxidase and 4-chloro-1-naphthol; 5, staining with biotinylated SNA lectin, streptavidin-alkaline phosphatase conjugate and X-phosphate/NBT; 6, staining with MAA lectin, streptavidin-alkaline phosphatase conjugate and X-phosphate/NBT.

1–3). Sialylation influenced the migration of DPP IV under these conditions in that after complete desialylation of DPP IV (Fig. 9, lane 2) with neuraminidase from *C. perfringens* the desialylated, less charged form migrated more slowly than sialylated DPP IV (Fig. 9, lane 1). After exposure to neuraminidase from the *Newcastle disease virus*, which had removed α2,3-sialic acid only, the DPP IV band (Fig. 9, lane 3) co-migrated with that one in lane 1, showing that no desialylation occurred in this case and native DPP IV from thymocyte plasma membranes exists exclusively in α2,6-sialylated form. This was also documented in the co-migrating band stained with biotin-labeled SNA lectin specific for α2,6-linked sialic acids (Fig. 9, lane 5). Moreover, no staining of DPP IV band with biotin-labeled MAA, the lectin specific for α2,3-linked sialic acids, was apparent (Fig. 9, lane 6). Staining with anti-CD26 mAb (Fig. 9, lane 4) confirmed DPP IV activity of CD26.

4. Discussion

Our experiments demonstrate a weak agonistic, GC-like effect of AGs mifepristone and onapristone resulting in reduction of thymus mass, number of thymocytes (Figs. 1 and 3) and number of cycling cells in the thymocyte population (Table 2) in infant male rats. This effect is dose-dependent (Figs. 1 and 3) and limited to the pre-weaning period between the 12th and 17th postnatal days (Tables 1 and 2). To explain such an observation, not only glucocorticoid receptors (GRs) in the thymus but also GRs in the pituitary, which are involved in the feedback inhibition of adrenal secretion of GCs must be considered as potential targets of AG activity in vivo.

Desinhibition of the HPA axis after mifepristone application resulting in increased GC adrenal secretion has been described in women [24,25] and in lean adult Zucker rats with genetically determined obesity [26]. In humans, such desinhibition is dependent on circadian rhythm [24,27]. Our results presented in Table 3 might support such a mechanism, too. However, several other of our findings testify against the increased adrenal secretion as a factor responsible for the moderate thymolytic effect of AGs: (1) at the same doses, both AGs used were capable of antagonizing the effect of exogenously administered HC on the thymus mass and thymocyte counts (Figs. 1 and 2) as well as on major thymocyte subset proportions as visualized by CD4, CD8 and CD26 expression (Figs. 4–6). (2) The agonistic effect of mifepristone was observed between the 12th and 17th postnatal day, when the plasma corticosterone level is low and starts to rise [3], whereas it was missing during the weaning period (19th to 26th day, Tables 1 and 2) characterized by higher levels of corticosterone in blood circulation [2,3].

We thus had to look for the explanation of the weak agonist action of AGs at the level of GRs in the thymus [13]. Besides the fact that plasma corticosterone level is low in 12-day-old rats [3] a relatively high intrinsic steroid production observed in fetal and neonatal murine thymus had to be taken into account [28].

The role of adrenal GCs in the postnatal involution of thymus and thymocyte death under stressing conditions is generally accepted [1,2,29,30–32]. In rats, thymus involution coincides with the postweaning period 4 weeks after birth. It is interesting that we could not demonstrate hypertrophy of the thymus on day 26 after three daily doses of mifepristone (Table 1), which would simulate adrenalectomy as described by Inomata and Nakamura [2], although we could observe a positive effect on cycling thymocytes on day 19 (Table 2). The doses used in this experiment are comparable with those applied in Figs. 1 and 2, where the antiglucocorticoid effect on thymus mass and thymocyte count was demonstrated. The possible explanation for not being able to demonstrate hypertrophy of the thymus (Table 1) could be an incomplete blockage of thymic GRs by mifepristone. The relative binding affinities of mifepristone and onapristone to rat thymus cytosol GRs were of values 50 and 53, respectively, compared with value 100 for synthetic GC dexamethasone [13]. However, we cannot be sure about the effect of AGs in vivo on circulating corticosterone as the natural hormone of the rat (see Section 1).

The slight thymolytic effect of AGs in rats 12–17-day-old (when corticosterone secretion is low) and their antagonistic effect to exogenously administered HC would be consistent with the "mutual antagonism" model of thymocyte selection and the role of presumed GCs produced in thymic epithelial cells [28,33], which would provide protection against thymocyte deletion (Ashwell et al. [8], Vacchio and Ashwell [33]). Their protective effect could be abolished by AGs. The "mutual antagonism" model suggests that GCs would induce thymocyte apoptosis at high concentrations but, at lower concentration, thymic GCs could antagonize TCR-mediated deletion signals and allow for survival of thymocytes [34,35].

Another explanation would require direct competition for thymic GRs between AGs as weak agonists and GCs. A limited agonistic mechanism of mifepristone action has been proposed by Spitz and Bardin [36]. In postmenopausal women and ovarectomized monkeys, mifepristone acts as a progestin agonist. Mifepristone inhibits the actions of progesterone when the latter is present (see [36]). These observations would have their analogy in the GC-like activity of AGs in infant rats, which have low GC secretion [3].

A latent agonist activity of mifepristone in fibroblasts treated with activators of protein kinase A has been reported by Nordeen et al. [37], in contrast to the action of onapristone [37], and another progesterone receptor antagonist ZK 230211 Schering [38], which were devoid of such a latent agonist effect on protein kinase A.

As shown in previous studies [9], GCs induce rapid apoptosis in immature cortical DP thymocytes in vivo. Here we demonstrate the inhibition of this process by GC receptor antagonists mifepristone and onapristone. In contrast to AG agonistic effect on thymus weight and thymocyte counts in vivo, no impact of AG administration on DP thymocyte proportions has been observed (Figs. 4 and 5). This is in agreement with the results of others [9] showing that mifepristone treatment of transgenic mice did not significantly influence the percentage of $CD4^+CD8^+$ cells in the thymus. In addition, while mifepristone protected thymic organ cultures from the thymolytic effect of dexamethasone, it did not influence thymocyte counts when used alone [9]. Thus, the agonistic effect of AGs in vivo seems to be a part of another mechanism.

CD26 is a 105–110 kDa glycoprotein identified as dipeptidyl peptidase IV (EC 3.4.14.5) [23]. CD26 was originally described as a T-cell activation marker [39,40]. However, serine aminopeptidase DPP IV activity is widely distributed in various tissues and cell types [41,42] and it also exists in a soluble form in serum [43]. CD26 upregulation in T cells is known to occur upon antigen-specific or polyclonal activation [44,45]. High CD26 expression has also been assigned to the T helper 1 (Th 1) lymphocyte population [46,47].

In order to characterize HC and AGs effects on thymic CD26/DPP IV in young rats we have performed FCM analysis of CD26 expression on the surface of thymocyte subsets and determined total DPP IV activity. We have also studied DPP IV activity in IEF fractions to evaluate the contribution of sialylation to the heterogeneity of DPP IV. On the whole, we have observed a relative increase of $CD26^{hi}$ thymocyte numbers (Fig. 6C) and overall DPP IV activity after HC injection to suckling rats. When individual thymocyte subsets were analyzed separately, a drop in DP cell numbers was apparent upon HC administration, which was accompanied by the increase of the earliest, large CD3-negative precursor stages of thymopoiesis. As $CD3^-$ thymocytes express the highest surface level of CD26 (Fig. 6A) and downregulation of CD26 has been documented in apoptotic DP cells [48], our findings of HC-mediated changes of CD26 expression and activity go well together. We can speculate that CD26/DPP IV of the earliest precursor cells, an enzyme known to possess binding capacity for certain proteins of extracellular matrix [49], could be a mediator of thymocyte migration from the periphery to the thymus. The reversal of HC effect by mifepristone concerned mainly CD26 expression on this population (Fig. 6C), the overall DPP IV activity of thymocyte subpopulation mixture being not changed by mifepristone administration (Fig. 7). This may be due to the lack of correlation between CD26 surface expression and other membrane-bound and cytoplasmic forms of DPP IV-like enzyme activities [48,50,51]. Thus, the cell-surface 75–80 kDa protein referred to as DPP IV-β and coexpressed with CD26 [52,53] was characterized in T lymphoblastoid cells. Another DPP IV activity, a membrane-bound 175 kDa protein, was identified as human serum protein attractin [54–56]. Abbott et al. [57,58] also described a 100 kDa DPP IV homologue DPP8 in the cytoplasm of human T-cells. Recently structure/function relationships of various molecules with DPP IV-like activity have been reviewed by Šedo and Malík [59].

The heterogeneity of DPP IV activity in rat thymocyte membrane fraction was demonstrated by IEF of samples digested by *C. perfringens* neuraminidase. The sialylated acidic components of DPP IV activity after neuraminidase treatment did not shift to one more basic pI but to some three groups of closely related pIs (Fig. 8). Therefore, although sialylation contributes to the heterogeneity of DPP IV activity, as confirmed also for intestinal [19], kidney and liver [60] enzyme in rats, the principal reason for different pI values of DPP IV does not seem to be due exclusively to sialylation.

IEF analysis (Fig. 8) has revealed that all membrane-bound forms of DPP IV are sialylated. It means that, in spite of significant differences in the level of CD26 expression among thymocyte subpopulations [48,61], both cortical and medullary thymocytes carry sialylated CD26. It is assumed that rat DPP IV/CD26 contains eight N-glycosylation sites [62], potential acceptor substrates for two sialyltransferases α2,6-[ST6(N)] and α2,3-[ST3(N)] with mRNA uniformly distributed throughout the thymus [63]. Our preliminary findings [64] indicate uniform expression of surface α2,3- and α2,6-sialic acids in rat thymocyte subpopulations as detected by MAA and SNA binding and flow cytometry.

However, N-glycans in some surface markers (CD45) have exclusively SAα2,6-sequences while in other glycoproteins (CD11a/CD18) only SAα2,3-sequences can be found [63]. The question is whether the sialylation pattern of DPP IV is not modified during thymocyte differentiation. The results of some authors [63] suggest that, in the initial stages of the differentiation pathway, human thymocytes express predominantly α2,3-linkages stained with MAA, while mature thymocytes stain with SNA. Other authors [65], however, published contradictory results showing that murine cortical thymocytes preferentially bound SNA and medullary cells were recognized by MAA. Using lectin affinity blotting (Fig. 9) we demonstrate only α2,6-linkages in native CD26/DPP IV in all thymocyte subsets in rats, which is similar to what was shown for liver and kidney DPPIV in this species [60]. It is possible that this type of sialylation participates in the hypersialylation of DPP IV from T cells of HIV-infected individuals reported by Smith et al. [66]. Surprisingly, in our experimental design we have not been able to show any changes of the α2,6-sialylation pattern that is known to be sensitive to the treatment with GC and AG [67,68]. One could speculate that immature $CD26^{hi}$ cells homing to the thymus are not sensitive to GC-mediated modulation of α2,6-sialyltransferase activity.

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