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Suppression of PrP^{Sc}- and HIV-1 gp120 induced neuronal cell death by sulfated colominic acid

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The scrapie prion protein (PrP^{Sc}) has been shown to induce apoptosis of rat cortical neurons *in vitro*. Here we demonstrate that the toxic effect displayed by PrP^{Sc} can be blocked by sulfated colominic acid (polymer of *N*-acetylneuraminic acid). This compound acts neuroprotectively at a concentration of $\geqslant$0.3 μg/ml when preincubated with the neurons or PrP^{Sc}. Rat cortical cells also undergo apoptosis after incubation with the HIV-1 coat protein gp120 *in vitro*. This effect was abolished also by sulfated colominic acid when preincubated with the cells or gp120. Addition of 0.3 μg/ml of compound resulted in an increase in cell viability by about 1.6–1.9-fold compared to cultures incubated for 18 h with 30 ng/ml of PrP^{Sc} or 20 ng/ml of gp120 alone (containing about 40% viable cells). Sulfated colominic acid does not act as antagonist of NMDA receptor channels at concentrations of up to 3 μg/ml when co-administered with 100 μg/ml of NMDA. It displayed a strong cytoprotective effect on human T lymphoblastoid CEM cells exposed to HIV-1; a 50% protection occurred after preincubation of the cells with 0.43 μg/ml of compound. At the same concentration, the compound caused an inhibition of HIV-1-induced syncytium formation. Sulfated colominic acid may be a promising compound for treatment of dementia caused by PrP^{Sc} and HIV-1 infections.

Keywords: prion protein; HIV-1; gp120; sulfated colominic acid; neurotoxicity; NMDA receptor; neurons; CEM cells

Introduction

The scrapie prion protein (PrP^{Sc}) is able to induce apoptosis in neuronal cells–both *in vitro* (Müller *et al*, 1993) and *in vivo* (Fairbairn *et al*, 1994; Giese *et al*, 1995; Lucassen *et al*, 1995). PrP^{Sc} is considered as the causative agent of transmissible neurodegenerative diseases, including Creutzfeldt-Jakob disease and Gerstmann-Sträussler syndrome in humans, and scrapie and bovine spongiform encephalopathy in animals (Prusiner, 1994). This deleterious protein is thought to be formed by conversion of the putative α-helices of the normal cellular prion protein (PrP^{C}) into β-sheets (Gasset *et al*, 1992). The mechanism underlying the neurotoxic effect of PrP^{Sc} and of its peptide fragment, PrP 106–126 (Forloni *et al*, 1993; Perovic *et al*, 1995) is not yet fully understood. PrP^{C} is present predominantly in the brain, where it may play a role in synaptic function (Collinge *et al*, 1994), but lower amounts of this protein have also been detected in other tissues (Bendheim *et al*, 1992). PrP^{C} may contribute to the integrity of synapses containing GABA receptors (Collinge *et al*, 1994) and has been shown to increase the synaptosomal levels of intracellular free calcium through an interaction with voltage-sensitive calcium channels (Whatley *et al*, 1995). The PrP^{Sc} has been shown to affect ionotropic glutamate receptors, especially the *N*-methyl-D-aspartate (NMDA) receptor; this effect can be prevented by NMDA receptor antagonists (Müller *et al*, 1993). The PrP^{Sc}-induced neurotoxicity seems to be associated with the production of reactive oxygen species (ROS), resulting in apoptosis of the cells (Schröder *et al*, 1999). Furthermore, the PrP 106–126 peptide has been shown to decrease the levels of intracellular glutathione in neuronal cells (Perovic *et al*, 1996, 1997).

At present, only a few drugs are known which may protect animals against PrP^{Sc} infection and suppress PrP^{Sc}-induced neurotoxicity: (i) polyanio-

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nic glycans, including pentosan sulfate and dextran sulfate, have been shown to prolong the incubation period and to inhibit PrP^{Sc} formation in rodents (Diringer and Ehlers, 1991), most likely by reducing of the amount of PrP^{C} on the cell surface through enhanced endocytosis of PrP^{C} (Shyng *et al*, 1995); (ii) the polyene macrolide antibiotic, amphotericin B (Pocchiari *et al*, 1989), and its derivative, MS-8209 (Adjou *et al*, 1995), also cause a prolongation of the incubation period of PrP^{Sc}-infected mice; (iii) the accumulation of PrP^{Sc} could also be blocked by Congo red (Caughey and Race, 1992) and (iv) the anthracycline 4′-iodo-4′-deoxy-doxorubicin, which bind to amyloid fibrils (Tagliavini *et al*, 1997); (v) the neutotoxic effect displayed by PrP^{Sc} was found to be prevented by antagonists of NMDA receptor channels, such as memantine and MK-801 (Müller *et al*, 1993; Perovic *et al*, 1995; Brown *et al*, 1996); and (vi) the triaminopyridine flupirtine, a clinically used non-opiod analgesic drug, was found to display *in vitro* a strong cytoprotective effect on neurons treated with PrP^{Sc} or its toxic fragment, PrP 106–126 (Perovic *et al*, 1994, 1995). This drug enhances the level of the antiapoptotic protein Bcl-2 and normalizes the level of intracellular glutathione (Perovic *et al*, 1996, 1997).

Apoptosis is thought also to be one major mechanism responsible for the cytopathic effect of the human immunodeficiency virus type 1 (HIV-1), resulting in the progressive loss of neurons and lymphocytes (in particular $CD4^{+}$ lymphocytes) during the progression of the disease (Gougeon *et al*, 1993; Schattner and Laurence, 1994). We described that neurons undergo cell death following exposure to the isolated viral glycoprotein gp120 *in vitro* (Müller *et al*, 1992). This deleterious effect could be prevented, like PrP-induced apoptosis, by the NMDA receptor channel blockers, memantine and MK-801 (Müller *et al*, 1992). Gp120 is known to bind to the surface glycoprotein CD4, which is expressed on a subset of T-cells and macrophages (Sattentau and Weiss, 1988). The binding site for gp120 at the neurons is not known. Gp120 does not interact with the NMDA receptor directly (Ushijima *et al*, 1993). We found that exposure of neuronal cells to gp120 causes an activation of phospholipase A_2 resulting in an increased release of arachidonic acid which in turn may sensitize the NMDA receptor (Ushijima *et al*, 1995). Recent reports indicate that gp120-mediated apoptosis of neuronal cells can be prevented by compounds which lower the load of ROS (Müller *et al*, 1997).

Until now there are no effective therapies for treatment of apoptosis induced by PrP^{Sc}, or gp120. Recently it has been reported that sulfated colominic acid exhibits anti-HIV-1 activity (Yang *et al*, 1996). Colominic acid is a homopolymer of *N*-acetylneuraminic acid, containing α-2,8 ketosidic linkages between the sugar moieties (McGuire and Binkley, 1964). Here we show that sulfated colominic acid (Figure 1) is a potent inhibitor of both PrP^{Sc} and gp120-induced neuronal cell death.

Results

Neuroprotective effect of sulfated colominic acid

Neuroprotective effect on PrP^{Sc}-treated neuronal cultures Treatment of rat cortical cells with 30 ng/ml of PrP^{Sc} entrapped into liposomes for 18 h resulted in a significant ($P<0.01$) reduction of cell viability compared to untreated control (decrease in $A_{595\ nm}$ value by 51% in MTT assay; Figure 2B). In parallel with the decrease in cell viability, a strong and significant ($P<0.01$) increase in apoptosis (by 4.6-fold based on $A_{405\ nm}$ value in cell-death detection ELISA which measures the presence of soluble histone-associated DNA fragments) was observed in the presence of 30 ng/ml of PrP^{Sc} compared to the assay in the absence of PrP^{Sc} (Figure 2A). No significant effect on both apoptosis (Figure 2A) and cell viability (Figure 2B) was observed using a detergent liposome extract, which was prepared from a pelleted fraction from uninfected normal brain.

Addition of sulfated colominic acid partially abolished the cytotoxic effect displayed by PrP^{Sc}. After preincubation with the compound for 30 min, the viability of the cells significantly ($P<0.01$) increased from 42% ($A_{595\ nm}$ 0.28; no compound) to 65% ($A_{595\ nm}$ 0.44; cultures treated with 0.3 μg/ml of sulfated colominic acid) compared to control cultures without PrP^{Sc} and compound ($A_{595\ nm}$ 0.67; set at 100%; Figure 3B). At higher concentrations, 1 or 3 μg/ml of sulfated colominic acid, the drug-mediated cytoprotection became >80% (Figure 3B). Pretreatment of the PrP^{Sc}-containing liposomes with 1 μg/ml of sulfated colominic acid (5 h, 4°C) prior to addition to the neurons also resulted in a significant ($P<0.01$) cytoprotective effect; increase in cell viability ($A_{595\ nm}$ value) from 0.32 ± 0.06 to 0.55 ± 0.06 ($47\pm8\%$ to $80\pm9\%$, compared to untreated control; n=10; not shown in Figure 3B). The cytoprotective effect of sulfated colominic acid could also be observed when the extent of apoptosis of the cells (presence of histone-associated DNA

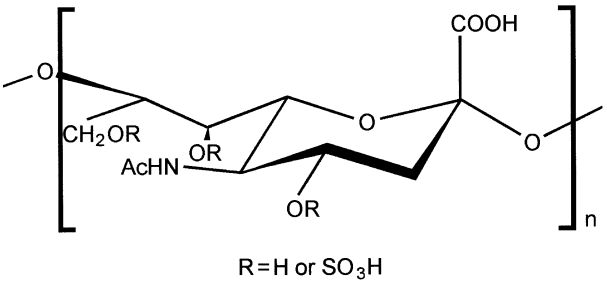

Figure 1 Structure of sulfated colominic acid.

fragments in the cytoplasm) was determined. The sulfated colominic acid reduced the increase in apoptosis caused by 30 ng/ml of PrP^{Sc} from $A_{405\ nm}$ 0.52 (absence of compound) to $A_{405\ nm}$ 0.39 (pre-incubation of the neurons with 0.3 μg/ml of sulfated colominic acid) compared to $A_{405\ nm}$ 0.11 (untreated control; Figure 3A). At higher concentrations, 1 and 3 μg/ml, the effect of sulfated colominic acid was even more pronounced; decrease in apoptosis to $A_{405\ nm}$ 0.23 and $A_{405\ nm}$ 0.19, respectively. A sig-

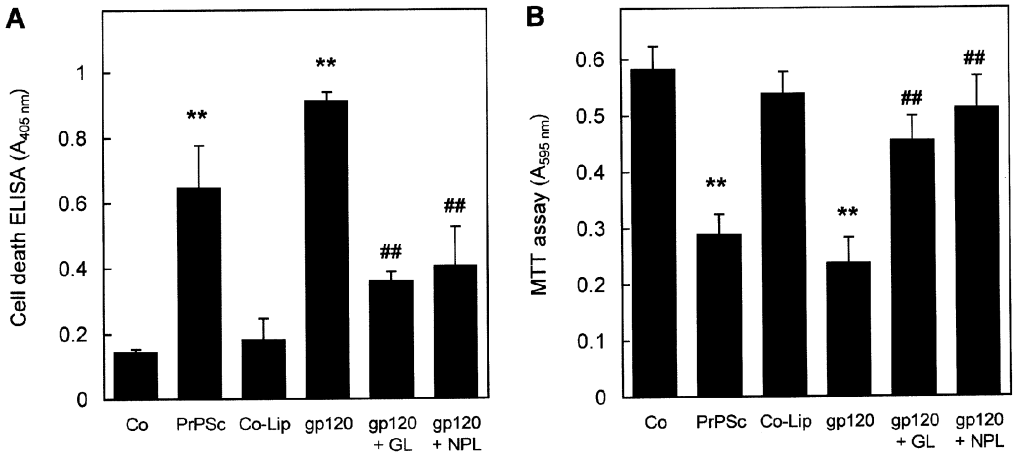

Figure 2 Induction of neuronal cell death by PrP^{Sc} and gp120 *in vitro*. The cultures remained either untreated (= Co) or were treated with PrP^{Sc}-containing detergent liposome preparation (30 ng/ml of PrP^{Sc}) (= PrPSc), or a detergent liposome extract, prepared from the pelleted fraction from an equivalent amount of uninfected brain lacking PrP^{Sc}, used as a negative control (= Co-Lip). Further cultures were treated with gp120 (20 ng/ml) in the absence (= gp120) or presence of 100 ng/ml of the lectin from *Gerardia savaglia* (= gp120+GL) and *Narcissus pseudonarcissus* (= gp120+NPL). After a total incubation period of 18 h apoptosis (presence of histone-associated DNA fragments in the cytoplasm) was determined by the cell-death detection ELISA (**A**) and viability was assayed using the MTT assay (**B**). The development of color in the peroxidase substrate buffer of the ELISA was determined photometrically by measuring the absorbance values at 405 nm; the formation of the formazan dye in MTT assay was monitored at 595 nm. The means $\pm$ s.d. of ten independent experiments are shown. The statistical significance of changes was analyzed with a one-way ANOVA, followed by Scheffé's test; $^{**}P<0.01$, compared with untreated control cultures; $^{\#\#}P<0.01$, compared with cultures treated with gp120 alone.

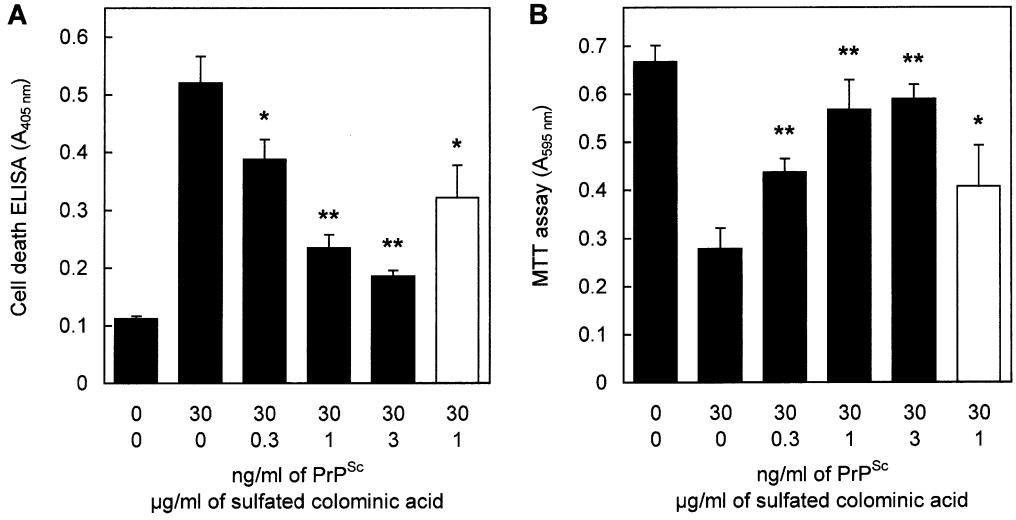

Figure 3 Neuroprotective effect of sulfated colominic acid on PrP^{Sc}-induced cytotoxic effect on rat cortical cells *in vitro*. The cells were incubated in the absence or presence of PrP^{Sc}; where indicated different concentrations of sulfated colominic acid were added to the cultures 30 min prior to the addition of PrP^{Sc} (filled bars). In one experiment, the sulfated colominic acid (1 μg/ml) was added 2 h after addition of PrP^{Sc} (open bar). After a total incubation period of 18 h apoptosis (presence of histone-associated DNA fragments in the cytoplasm) was determined by the cell-death detection ELISA (**A**) and viability was assayed using the MTT assay (**B**). Ten independent experiments were performed; means $\pm$ s.d. $^{*}P<0.05$, $^{**}P<0.01$ (one-way ANOVA and Scheffé's test; compared with PrP^{Sc}-treated cultures without sulfated colominic acid).

nificant ($P<0.01$) reduction of apoptosis (from $A_{405\ nm}$ 0.46 ± 0.04 to $A_{405\ nm}$ 0.26 ± 0.03; n=10) was also observed following exposure of cells to a PrP^{Sc} preparation that had been pretreated with 1 μg/ml of sulfated colominic acid, instead of untreated PrP^{Sc} (not shown in Figure 3A).

The sulfated colominic acid was found to be fully active only if the cells or PrP^{Sc} were preincubated with the compound. Addition of 1 μg/ml of sulfated colominic acid to the cultures 2 h after addition of PrP^{Sc} resulted in a decrease in apoptosis to only $A_{405\ nm}$ 0.32 ($P<0.05$; Figure 3A) and an increase in viability to only $A_{595\ nm}$ 0.41 ($P<0.05$; Figure 3B).

The PrP^{Sc}-induced cytotoxicity was completely abolished if the preparation was preincubated with the R073 antibody (ratio PrP^{Sc} : R073, 15 μg : 2500 μg) prior to the addition to the neurons; the antibody alone did not affect the viability of the cells (not shown; see also Müller *et al*, 1993).

The effect of sulfated colominic acid alone on the neuronal cells is shown in Figure 4. The results revealed that, in the concentration range tested, the compound does not significantly affect the naturally occurring neuronal cell death, as determined in cell-death detection ELISA (Figure 4A) and MTT assay (Figure 4B).

Treatment of neuronal cell cultures with PrP^{Sc} or sulfated colominic acid might result in glial cell proliferation. Therefore, the cellular composition of the cultures was determined before and after the test period. However, the results revealed that the content of glial cells did not markedly change during the incubation period (18 h) of the cultures with the PrP^{Sc} liposome preparation (30 ng/ml of PrP^{Sc}) or sulfated colominic acid (3 μg/ml); the percentage of GFAP-positive cells in the cultures amounted to 4–6%.

Neuroprotective effect on gp120-treated neuronal cultures Rat cortical cells undergo apoptosis also following incubation with the HIV-1 coat protein gp120 *in vitro*. At a concentration of 20 ng/ml, gp120 increased the amount of soluble DNA-histone complexes (apoptosis) by about 5–6-fold compared to untreated control ($P<0.01$; Figures 2A and 5A); simultaneously the cell viability ($A_{595\ nm}$ value) decreased by about 60% (Figures 2B and 5B). Also, this effect was abolished by addition of sulfated colominic acid. Preincubation of the cultures with 0.3 to 3 μg/ml of sulfated colominic acid resulted in a significant ($P<0.01$) reduction of the gp120-induced apoptosis (decrease in the amount of soluble DNA-histone complexes ($A_{405\ nm}$ values) by 53–68%; Figure 5A). Concomitantly with the decrease in apoptosis in the assays with sulfated colominic acid, the cell viability ($A_{595\ nm}$ value) significantly ($P<0.01$) increased from 41% to 78–90% of $A_{595\ nm}$ value of untreated control without gp120 (Figure 5B). A significant ($P<0.01$) reduction of gp120-induced cytotoxicity was also found following pretreatment of gp120 with sulfated colominic acid (1 μg/ml); apoptosis: decrease in $A_{405\ nm}$ value of gp120-treated cultures from 0.80 ± 0.19 to 0.38 ± 0.04 ($A_{405\ nm}$ value of control cultures without gp120, 0.18 ± 0.03; n=6); cell viability: increase in $A_{595\ nm}$ value of gp120-treated cultures from 0.29 ± 0.04 to 0.48 ± 0.05 ($A_{595\ nm}$ value of control cultures without gp120, 0.60 ± 0.04; n=6; not shown in Figure 5A and B). Again, a lower neuroprotective effect was observed if the sulfated colominic acid (1 μg/ml) was added to the cells 2 h after addition of gp120 (Figure 5A and B).

As a control for the specificity of the interaction between gp120 and neuronal cells, antibodies against gp120 were added. The gp120-mediated

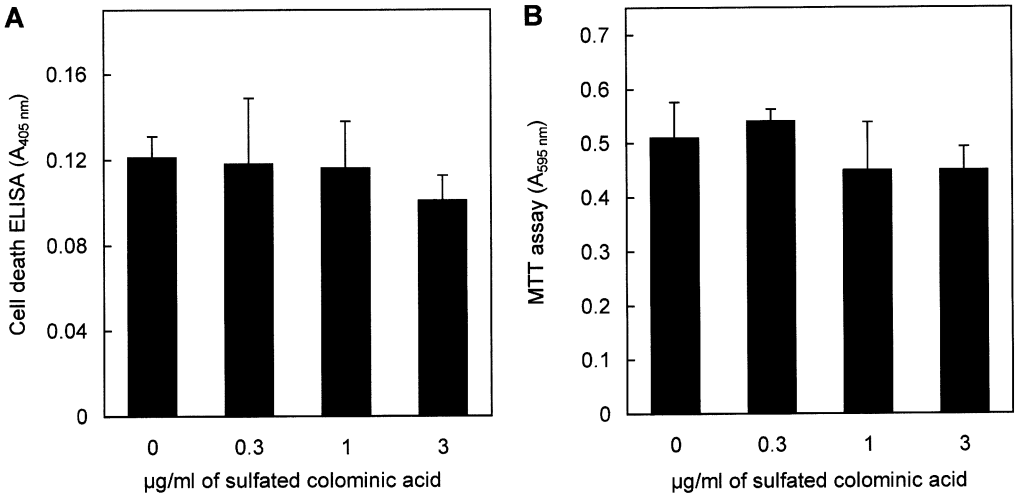

Figure 4 Effect of sulfated colominic acid alone on the presence of soluble histone-associated DNA fragments and cell viability in rat neuronal cultures. Cells were incubated for 18 h in the absence or presence of different concentrations of compound and analyzed by cell-death detection ELISA (**A**) and MTT assay (**B**). Ten independent experiments were performed; means ± s.d. Statistical analysis by one-way ANOVA and Scheffé's test revealed no significant effect ($P>0.05$).

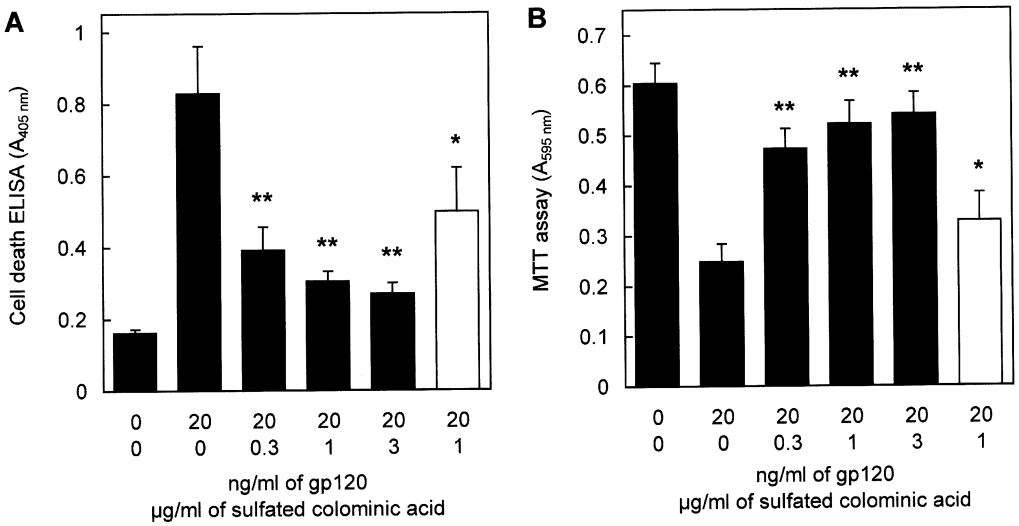

Figure 5 Neuroprotective effect of sulfated colominic acid on gp120-induced cytotoxic effect on rat cortical cells *in vitro*. Cells were preincubated with various concentrations of sulfated colominic acid for 30 min (37°C) and subsequently incubated in the absence or presence of gp120 for 18 h (filled bars). In one additional experiment, the sulfated colominic acid (1 µg/ml) was added 2 h after addition of gp120 (open bar). Apoptosis (presence of histone-associated DNA fragments in the cytoplasm) was determined by the cell-death detection ELISA (**A**) and viability was assayed using the MTT test (**B**). Six independent experiments were performed; means ± s.d. $*P<0.05$, $**P<0.01$ (one-way ANOVA and Scheffé's test; compared with gp120-treated cultures without sulfated colominic acid).

toxic effect could be completely abolished by the antibodies with respect to both DNA fragmentation (occurrence of extranuclear mono- and oligonucleosomes) and to cell viability (not shown; see also Ushijima *et al*, 1995).

Previously we reported that the lectin from *Gerardia savaglia* (GL) (M_r 32 000; Kljajic *et al*, 1987) and the lectin from *Narcissus pseudonarcissus* (NPL) (M_r 25 000; Van Damme *et al*, 1988), which bind to gp120 with high affinity (Müller *et al*, 1988), are able to suppress infection of cells with HIV-1 (Weiler *et al*, 1990; Müller *et al*, 1988) and gp120-induced neurotoxicity (Müller *et al*, 1992). As shown in Figure 2, coincubation of gp120 with a 19-fold (GL) or 24-fold (NPL) molar excess of the lectins compared to gp120 almost totally abolished both gp120-induced apoptosis ($P<0.01$; Figure 2A) and cell killing ($P<0.01$; Figure 2B).

Treatment of the cells with gp120 did not significantly affect glial cell proliferation during the incubation period; the percentage of GFAP-positive cells in the cultures before and after the incubation period (18 h) was 5% and 6%, respectively.

Effect on NMDA-caused neuronal cell death

The excitatory amino acids, glutamate and NMDA, are known to induce cortical neurons to apoptosis (Kure *et al*, 1991; Müller *et al*, 1992, 1993). These amino acids activate the NMDA receptor complex, a ligand-gated Ca^{2+} channel (Reynolds and Miller, 1990). Therefore, we determined the effect of the sulfated colominic acid on NMDA-induced neurotoxicity.

Table 1 Effect of sulfated colominic acid on NMDA-induced neurotoxicity

Pretreatment	*(µg/ml)*	*(µM)*	*NMDA (µg/ml)*	*Viable cells ($A_{595\ nm}$)*
None			0	0.73 ± 0.05
None			100	0.10 ± 0.02
Sulfated colominic	0.3		100	0.12 ± 0.02
acid:	1.0		100	0.14 ± 0.02
	3.0		100	0.10 ± 0.02
MK-801:		1	100	0.65 ± 0.06
		10	100	0.69 ± 0.03
Memantine		5	100	0.64 ± 0.01
		50	100	0.70 ± 0.05

Where indicated the neurons were pretreated with sulfated colominic acid, MK-801 or memantine for 30 min and subsequently incubated with 100 µg/ml of NMDA. The cells were analyzed for viability (n=6) as described under Materials and methods. The means ± s.d. are given.

As shown in Table 1, at a concentration of 100 µg/ml of NMDA the viability of the cells strongly decreased, as determined in MTT assay. If the neurons were pretreated with sulfated colominic acid, no significant alteration of the toxic effect on neurons caused by NMDA was observed (Table 1). From this result we conclude that sulfated colominic acid does not act as NMDA receptor antagonist at concentrations of up to 3 µg/ml when co-administered with 100 µg/ml of NMDA.

In contrast, pretreatment of the neurons with the non-competitive NMDA receptor antagonists, MK-801 (1 and 10 μM) and memantine (5 and 50 μM), abolished the NMDA-caused toxicity (Table 1).

Cytoprotective effect of sulfated colominic acid on HIV-1-infected CEM cells

As shown in Figure 6, the sulfated colominic acid exhibited a strong cytoprotective effect on HIV-1-infected CEM cells. Addition of 0.3 μg/ml and higher of sulfated colominic acid significantly protected the cells against the cytopathic effect of the virus (5-day incubation period); a 50% protection occurred after preincubation of the cells with 0.43 μg/ml of compound (Figure 6). This effect was only observed if the cells were pretreated with sulfated colominic acid (for 2 h) before infection, but not if the compound was added after infection with HIV-1 (not shown). The dose-response curves showed that the sulfated colominic acid was not toxic to the cells up to the maximum concentration tested of 10 μg/ml (Figure 6).

Increasing the infectious dose from 10 to 100 and 1000 virus particles per cell caused only a slight reduction of the cytoprotective effect of sulfated colominic acid after an incubation period of 5 days in MTT assay. The absorbance values at 595 nm in the absence or presence of 3 μg/ml of sulfated colominic acid amounted as follows. Uninfected cells; without compound: 1.02 ± 0.11, with compound: 0.97 ± 0.15; 10 virus particles per cell; without compound: 0.21 ± 0.10, with compound 0.95 ± 0.16; 100 virus particles per cell; without compound: 0.14 ± 0.09, with compound: 0.89 ± 0.17; and 1000 virus particles per cell: without compound: 0.10 ± 0.07, with compound: 0.80 ± 0.10 (n=3).

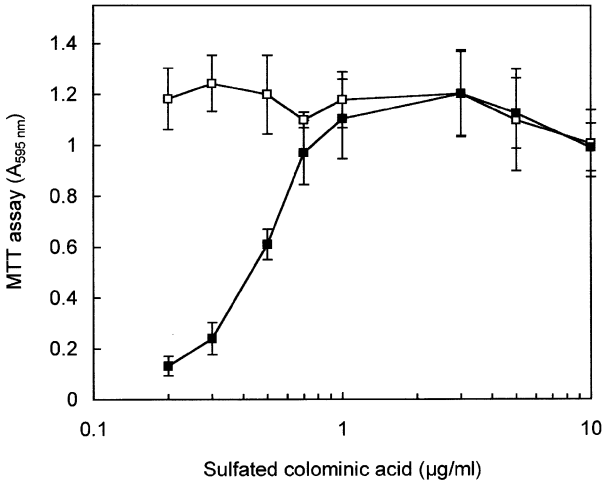

Figure 6 Effect of sulfated colominic acid on viability of HIV-1-infected CEM cells. The sulfated colominic acid was added to the cells 2 h prior to the addition of virus and was present throughout the 5-day incubation period. The viability of uninfected cells (□) and HIV-1-infected cells (■) was determined spectrophotometrically at 595 nm using MTT assay; the $A_{595\,nm}$ values of the cultures without compound at day 5 amounted to 1.19 ± 0.07 (uninfected cells) and 0.15 ± 0.04 (HIV-1-infected cells), respectively. Results are the means ($\pm$s.d.) from three independent experiments.

Inhibition of HIV-1-induced syncytium formation by sulfated colominic acid

Next cell fusion assays were performed to determine the anti-HIV activity of sulfated colominic acid. The results revealed that sulfated colominic acid efficiently abolished HIV-1-induced syncytium formation, following co-cultivation of uninfected Molt-3 cells with chronically HIV-1-infected Molt-4 cells. At a concentration of 0.3 μg/ml and higher of sulfated colominic acid, markedly less giant cells were found after 24 h, and those which had been formed, were smaller in size. The results were less pronounced when cells were observed after 48 h under the microscope (results not shown).

Discussion

Both PrP^{Sc} and the HIV-1 coat protein gp120 induce neurotoxicity *in vitro* (Lipton *et al*, 1991; Müller *et al*, 1992, 1993). Here we demonstrate that the toxic effect displayed by PrP^{Sc} and gp120 can be blocked by sulfated colominic acid. This compound acts neuroprotectively at a concentration of $\geqslant 0.3$ μg/ml when preincubated with the neurons or the toxic agents, PrP^{Sc} and gp120. The compound alone, at the concentrations used (up to 3 μg/ml), did not influence the naturally occurring neuronal cell death.

Previously we demonstrated that neuronal cell death induced by PrP^{Sc} or HIV-1 gp120 can be prevented by the NMDA receptor antagonists, memantine and MK-801 (Müller *et al*, 1992, 1993; Ushijima *et al*, 1993; Perovic *et al*, 1995). This result indicates that PrP^{Sc} and gp120 activate NMDA receptors, but the underlying mechanism is poorly understood. Gp120 does not interact with the receptor directly (Savio and Levi, 1993; Ushijima *et al*, 1993); it might act through macrophages, which are present in neuronal cultures at small amounts (Benos *et al*, 1994). Likewise, the PrP^{Sc}-induced activation of NMDA receptor might be caused by a production of NMDA-like neurotoxins through macrophages or an impairment of the release of growth factors from astrocytes. However, if the neurons were pretreated with sulfated colominic acid, no significant decrease in the toxic effect on neurons caused by NMDA was observed. Therefore we conclude that sulfated colominic acid does not act as NMDA receptor antagonist.

Sulfated colominic acid was found to display a strong cytoprotective effect on cells (human T lymphoblastoid CEM) exposed to HIV-1 *in vitro* in

the concentration range of 0.3 – 10 μg/ml, confirming previous results (Yang *et al*, 1996); a 50% protection occurred after preincubation of the cells with 0.43 μg/ml of compound. In the concentration range tested, the compound had no effect on cell growth. The fact that sulfated colominic acid exhibits cytoprotective activity only when preincubated with the cells but not when added to the cells after infection suggests that this non-toxic polyanion prevents the infection of the cells by inhibiting the adsorption of the virus. This assumption is also supported by the results obtained in the syncytium assay.

It has been shown that sulfated colominic acid does not affect the expression of CD4 antigen in T-cells (Yang *et al*, 1996); therefore this polymer has been proposed to bind to some other glycoproteins or glycolipids on the cell surface (Yang *et al*, 1996), e.g. CD26, a putative co-factor for the virus penetration into $CD4^+$ cells (Callebaut *et al*, 1993). Another target for this compound might be the V3 domain of HIV-1 gp120, which has been shown to bind sulfated polysaccharides (Batinic and Robey, 1992; Okada *et al*, 1995).

Sulfated colominic acid is a polyanion of sialic acid (*N*-acetyl-D-neuraminic acid) residues. Several polyanionic glycans exhibit inhibitory activity against prion and retroviral infections. These compounds, e.g. pentosan sulfate and dextran sulfate, have been shown to prolong the incubation time of prion infections in rodents (Diringer and Ehlers, 1991) and to inhibit PrP^{Sc} accumulation in neuroblastoma cells (Caughey and Raymond, 1993). The sulfated glycans stimulate endocytosis of PrP^c in cultured cells (Shyng *et al*, 1995). Therefore, these polymers may inhibit PrP^{Sc} propagation by reducing the amount of PrP^c on the cell surface. Dextran sulfate, heparin sulfate, chondroitin sulfate, and polysulfated polyxylan also inhibit infection of cells by HIV-1 (Baba *et al*, 1988; Biesert *et al*, 1988; Mitsuya *et al*, 1988). These compounds were found to abolish HIV-1-induced formation of syncytia and to block the adsorption of the virus to the $CD4^+$ target cells (Baba *et al*, 1988; Biesert *et al*, 1988). The sulfated polyglucan sulphoevernan, another polyanion displaying anti-HIV-activity *in vitro*, has been shown to act through binding to the virus (Weiler *et al*, 1990). Our results revealed that sulfated colominic acid only suppresses PrP^{Sc} and HIV-1 gp120-induced neurotoxicity when preincubated with these toxic agents. Thus, the mechanism of sulfated colominic acid resembles that of sulphoevernan, which inhibits HIV-1 infection by binding to viral gp120.

It is known that nonsulfated glycans are much less effective than the sulfated compounds (Caughey and Raymond, 1993). Consistently, the anti-HIV-1 activity of sulfated colominic acid was found to be much stronger than that of the non-sulfated polymer (Yang *et al*, 1996). Therefore, sulfated colominic acid and not the sulfur-free polymer was used in our experiments.

Both PrP^C and PrP^{Sc}, like gp120 (Geyer *et al*, 1988), undergo a number of post-translational modifications, including the attachment of asparagine-linked complex-type oligosaccharide side chains (Haraguchi *et al*, 1989). These oligosaccharides may represent potential binding sites for cellular carbohydrate-binding proteins serving as potential receptors for PrP^{Sc} (Schröder *et al*, 1994, 1998). Thus, the suppression of PrP^{Sc}-induced apoptosis by sulfated colominic acid may by due to an interference with the binding of PrP^{Sc} to these proteins. However, a competitive inhibition by sulfated colominic acid of the putative interaction between PrP and endogenous sulfated glycosaminoglycans found in the PrP amyloid (Caughey and Raymond, 1993) might be possible, too.

There is an urgent need to find out strategies for therapeutical intervention in human prion diseases, especially on the level of neuronal cell death caused by the infectious agent. Likewise, there are no effective treatments of the excessive cell death of neurons and lymphocytes in AIDS patients, a major hallmark of this disease (Ameisen, 1992). Due to its very low cytotoxicity, sulfated colominic acid may be a promising compound for a potential treatment of dementia in both prion and HIV-1 infections.

Materials and methods

Materials

NMDA, glycine, *p*-aminobenzoic acid, insulin, poly-L-lysine ($M_r > 300\,000$), cytosine arabinoside and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA); MK-801 [(+)-5-methyl-10,11-dihydro-5H- dibenzo[a,d] cyclophepten-5,10-imine maleate; dizocilpine] was from Research Biochemical International (Natick, MA, USA); L-glutamine, trypsin and polyclonal antibodies against gp120 (sheep) were from Biochrom (Berlin, Germany); and cell-death detection ELISA kit was from Boehringer Mannheim (Mannheim, Germany). The polyclonal antibody R073 (rabbit) against PrP 27 – 30 purified from scrapie-infected hamster brains (Serban *et al*, 1990) was a gift from Dr SB Prusiner (University of California, San Francisco). Memantine (1-amino-3, 5-dimethyladamantane) was a gift of Merz & Co. (Frankfurt/M, Germany).

The lectin from *Gerardia savaglia* (GL) and the lectin from *Narcissus pseudonarcissus* (NPL) were purified as described (Kljajic *et al*, 1987; Van Damme *et al*, 1988).

Sulfated colominic acid (Figure 1) was obtained from Marukin Shoyu Co. Ltd. (Kyoto, Japan). The sodium salt with an average molecular mass of 24 000 ($n \approx 50$) was used. The compound was

dissolved in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide at stock concentration of 10 mg/ml and stored at −20°C until use.

Preparation of PrP^{Sc} and HIV-1 gp120

PrP^{Sc} was purified from scrapie-infected hamster brain as described (Hilmert and Diringer, 1984; Sasaki *et al*, 1992). The PrP^{Sc} material was solubilized using a mixture of phosphatidylcholine and the nondenaturing detergent sodium cholate (Gabizon *et al*, 1987). The PrP^{Sc}-containing liposome material used contained 10 μg of PrP^{Sc} per milligram of phosphatidylcholine. For negative controls, a detergent liposome extract was prepared from the pelleted fraction from normal uninfected brain lacking PrP^{Sc} in the same way but without proteinase K digestion.

The gp120 was purified from HIV_{IIIB}-infected H9 cells (Popovic *et al*, 1984) as described (Robey *et al*, 1986; Matthews *et al*, 1987). The preparation was >95% pure as checked by polyacrylamide gel electrophoresis (Müller *et al*, 1988, 1992).

Neuronal cells

Rat primary cortical cells were prepared from the brains of 18-day-old Wistar rat embryos as described previously (Perovic *et al*, 1994, 1995). Briefly, after isolation cerebral hemispheres were placed into Hank's balanced salt solution (Biochrom) without Ca^{2+} and Mg^{2+}. Brain tissue was dissociated using 0.025% of trypsin (10 min; 37°C); the proteolytic reaction was stopped by addition of 10% fetal calf serum. The single cell suspension was centrifuged and the pellet containing dissociated neuronal cells was resuspended in Dulbecco's modified Eagle's medium (Sigma), supplemented with 30 mM glucose, 24.5 mM KCl, 7 μM *p*-aminobenzoic acid, 100 mU/l of insulin, 26 mM $NaHCO_3$, 2 mM L-glutamine and 10% fetal calf serum. The cells were seeded into poly-L-lysine (5 μg/ml; 200 μl/cm^2) coated plastic dishes (Nunc) at a concentration of 2×10^5 cells/cm^2. The cells were kept in an atmosphere of 95% air and 5% CO_2. To enrich the percentage of neurons the cultures were incubated for 24 h in the presence of 10 μM cytosine arabinoside in order to inhibit glial cell proliferation. Afterwards the cells were maintained in cytosine-arabinoside-free medium/serum. The cultures contained >90% of neurons (immunostaining with anti neurofilament 68 kDa as marker for neurons); the other cells were astrocytes (immunostaining with anti-glial fibrillary acidic protein, GFAP, for glial cells; Boehringer detection kit) and macrophages (as identified by expression of receptors for the Fc portion of immunoglobulin; Raff *et al*, 1979). The viability of the cells was >85% as determined by the trypan blue dye exclusion method. One day later the cells were used for the experiments.

Treatment of neuronal cells

Treatment with PrP^{Sc} The cultures were treated with 30 ng/ml of PrP^{Sc}, entrapped in liposomes, in growth medium/serum. Where indicated, sulfated colominic acid was added to the cultures 30 min prior to the addition of PrP^{Sc}. Alternatively, PrP^{Sc} pretreated with sulfated colominic acid was used (obtained by preincubation of PrP^{Sc}-containing liposomes with 1 μg/ml of sulfated colominic acid for 5 h at 4°C). The sulfated colominic acid and PrP^{Sc} were present throughout the total incubation period of 18 h. In control experiments, a detergent liposome extract from the pelleted fraction from uninfected brain lacking PrP^{Sc} was used instead of the PrP^{Sc}-containing liposomes, or the PrP^{Sc} preparation was preincubated (12 h, 4°C) with the anti-PrP^{Sc} antibody, R073, prior to use in the assay.

Treatment with gp120 The neuronal cells were treated with 20 ng/ml of gp120 in Ca^{2+}- and Mg^{2+}-free PBS containing 25 mM Tris/HCl, pH 7.4, and 15 mM glucose for 2 h at 37°C. The sulfated colominic acid was added 30 min prior to the viral protein. After incubation PBS was removed and replaced by growth medium/serum containing the same amount of gp120 and sulfated colominic acid; incubation proceeded for up to 18 h. In a second set of experiments, gp120 (150 ng/ml) was pretreated with 1 μg/ml of sulfated colominic acid for 5 h at 4°C prior to addition to the cells. In control experiments, HIV-gp120 (130 ng/ml) was reacted (2 h, 4°C) with antibodies against gp120 (10 μg/ml) prior to use in the assay (Müller *et al*, 1992).

Treatment with NMDA The neuronal cultures were treated (2 h, 37°C) with 100 μg/ml of NMDA (0.68 mM) including 10 μM glycine in Ca^{2+}- and Mg^{2+}-free PBS containing 25 mM Tris/HCl, pH 7.4, and 15 mM glucose. Where indicated, the cultures were pretreated with sulfated colominic acid, MK-801 or memantine for 30 min before NMDA was added. Following NMDA treatment, PBS was removed and replaced by growth medium/serum; incubation proceeded for 18 h.

CEM cells and virus infection

Human T lymphoblastoid CEM cells were maintained in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 in air atmosphere. The cells (5×10^5, in 1-ml assays) were infected with 5×10^6 HIV-1 particles (strain HIV_{IIIB} produced by chronically infected Molt-4 cells) and cultivated for up to 5 days. The sulfated colominic acid was added at a final concentration of 0.2 to 10 μg/ml 2 h prior to addition of the virus (= day 0) and was present throughout the experiment.

Cell fusion assay
Uninfected Molt-3 cells (T-lymphoblastoid cell line; 1.8×10^5 cells/assay) were co-cultured with chronically HIV-1-infected Molt-4 cells (3.75×10^5 cells/assay) in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence or the absence of 0, 0.1, 0.3, 1.0 and 3.0 μg/ml of sulfated colominic acid for 24 or 48 h. The formation of syncytia (defined as >4 nuclei within a common cell membrane) was semiquantitatively scored (Lifson *et al*, 1986).

Apoptosis assay
The photometric enzyme immunoassay, 'cell-death detection ELISA', which measures apoptosis by quantitating cytoplasmic histone-associated DNA fragments was used. For the assay, the cells were collected by centrifugation at $1000 \times g$ at room temperature for 10 min. The resulting pellets were treated with lysis buffer according to the instructions of the manufacturer (Boehringer Mannheim) and centrifuged again. The supernatants of the second centrifugation step were assayed for protein (see below) and equalized amounts of supernatants were analyzed for the presence of mono- and oligonucleosomes in the ELISA. Measurements were performed at 405 nm against substrate solution as a blank. The background values (incubation buffer instead of sample solution) were subtracted.

Cell viability
The viability of total cells was determined using the MTT colorimetric assay (Scudiero *et al*, 1988), followed by evaluation with an ELISA reader (BioRad 3550, equipped with the program NCIMR IIIB).

Protein concentration
Protein concentrations were estimated by the method of Lowry *et al* (1951) with bovine serum albumin as a standard.

Statistical analysis
The data are presented as the means $\pm$ s.d. For the statistical comparisons, one-way analysis of variance (ANOVA), followed by Scheffé's test for multiple comparison of means was used (Sachs, 1997). P values <0.05 were considered to be significant.

Abbreviations

GL, lectin from *Gerardia savaglia*; HIV-1, human immunodeficiency virus type 1; NMDA, *N*-methyl-D-aspartic acid; NPL, lectin from *Narcissus pseudonarcissus*; PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein.

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