JOURNAL OF VIROLOGY, Feb. 1993, p. 643–650
0022-538X/93/020643-08$02.00/0

Vol. 67, No. 2

Sulfated Polyanion Inhibition of Scrapie-Associated PrP Accumulation in Cultured Cells

BYRON CAUGHEY* AND GREGORY J. RAYMOND

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute for Allergy and Infectious Diseases, Hamilton, Montana 59840

Received 15 September 1992/Accepted 27 October 1992

The accumulation of an abnormal, protease-resistant form of the protein PrP (PrP-res) in hosts with scrapie and related transmissible spongiform encephalopathies appears to be important in disease pathogenesis. To gain insight into the mechanism of PrP-res accumulation and the in vivo antiscrapie activity of certain polyanions, we have studied effects of sulfated glycans on PrP metabolism in scrapie-infected neuroblastoma cells. Pentosan polysulfate, like the amyloid-binding dye Congo red, potently inhibited the accumulation of PrP-res in these cells without apparent effects on the metabolism of the normal isoform. The inhibition was due primarily to prevention of new PrP-res accumulation rather than destabilization of preexisting PrP-res. PrP-res accumulation remained depressed in the cultures after removal of the inhibitors. The activities of other sulfated glycans, nonsulfated polyanions, dextran, and DEAE-dextran were compared with those of pentosan polysulfate and Congo red. This comparison provided evidence that the density of sulfation and molecular size are factors influencing anti-PrP-res activity of sulfated glycans. The relative potencies of these compounds corresponded well with their previously determined antiscrapie activities in vivo, suggesting that the prophylactic effects of sulfated polyanions may be due to inhibition of PrP-res accumulation. Since PrP-res amyloid is known to contain sulfated glycosaminoglycans, we reason that these inhibitors may competitively block an interaction between PrP and endogenous glycosaminoglycans that is essential for its accumulation in a protease-resistant, potentially amyloidogenic state.

The transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases which include scrapie, Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, kuru, and bovine spongiform encephalopathy. In these diseases, an abnormal protease-resistant isoform of the endogenous protein PrP accumulates in the central nervous system and other tissues (7, 10, 18, 24, 35). Unlike the normal, protease-sensitive PrP (PrP-sen), the protease-resistant PrP (PrP-res) is insoluble in many detergents and can aggregate into amyloid-like plaques (5) and fibrils (24, 51) with high β-sheet content (17). Although the etiology of the TSEs is not clear, there is ample evidence that PrP plays an important role in the pathogenesis of these diseases (11, 12, 19, 20, 22, 25, 31, 32, 36–38, 54). In addition, it has been proposed that PrP-res is a component of the infectious agent, or the agent itself (7, 50), but this issue remains controversial (1, 19, 39, 55, 63).

The molecular basis for the difference between PrP-res and PrP-sen remains unknown. No general scrapie-specific covalent modifications of PrP have been identified that can account for the aberrant properties of PrP-res. Our studies with scrapie-infected mouse neuroblastoma cells (sc^+-MNBs) have shown that the formation of PrP-res occurs relatively slowly after its apparently normal PrP-sen precursor reaches the cell surface (15). Soon after its formation, PrP-res is exposed to lysosomal or endosomal proteases and truncated at the N terminus (16). Taken together, these studies indicated that the conversion of PrP to the protease-resistant state likely occurs at the plasma membrane or along an endocytic pathway to the lysosomes (13, 15, 16). Once formed, PrP-res is resistant to complete degradation (8, 15) and appears to accumulate in the secondary lysosomes (46).

Further studies of the mechanism of PrP-res formation, the relationship of PrP-res to pathogenesis and infectivity, and potential therapies for the TSEs would be aided by the availability of selective inhibitors of PrP-res accumulation. Recently, we found that Congo red, a dye that has long been used as a diagnostic stain for amyloids (30), potently inhibits the PrP-res accumulation in sc^+-MNBs without apparent effects on PrP-sen metabolism (14). The mechanism for the inhibition of PrP-res accumulation by Congo red is not known. However, since Congo red binds to amyloid fibrils of PrP-res (51), it appears likely this direct interaction could interfere with a critical event in PrP-res formation or destabilize the structure once it is formed (14). Although Congo red is a small disulfonated molecule (molecular weight [MW] of 697), it may be able to stack extensively and act as a sulfonated polyanion (61). Interestingly, prophylactic administration of certain polyanions has been shown to prolong the life span of animals inoculated with scrapie (23, 27–29, 40, 41, 43). Although there is evidence that the polyanions inhibit the early uptake and replication of the scrapie agent in the lymphoreticular system (23, 27–29, 40, 41) or in nerve endings (43), the prophylactic mechanism of these polyanions has not been defined at the cellular or molecular level. However, one consequence of the prophylactic treatment of scrapie-infected mice with pentosan polysulfate (PS) is a lack of PrP-res in brain tissue (23). Given these observations, it seems possible that a mechanism of action of the prophylactic polyanions may be an inhibition of PrP-res accumulation in scrapie-infected cells like that observed in vitro with Congo red (14).

In this report, we explore this possibility by analyzing the effects of sulfated glycans on PrP-res metabolism in sc^+-MNBs. We show that, like Congo red and with similar potency, certain sulfated glycans can selectively inhibit the accumulation of PrP-res. Thus, these inhibitors target a

* Corresponding author.

cellular event that is important for PrP-res formation or stabilization. Furthermore, this inhibition of PrP-res accumulation may account for the prophylactic antiscrapie activities of sulfated glycans in vivo.

MATERIALS AND METHODS

Chemicals. Congo red (98%) was from Aldrich. PS, iota-, lambda-, and kappa-carrageenans, dextran sulfates (average MWs of 8,000 and 500,000), heparin (MW of 4,000 to 6,000), polygalacturonic acid (sodium salt), and poly-L-glutamic acid (sodium salt; MW of 2,000 to 6,000) were purchased from Sigma. Dextran T500 and DEAE-dextran were purchased from Pharmacia, and amphotericin B (Fungizone) was purchased from Squibb. All were dissolved in water before being diluted into tissue culture medium. Recombinant *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PIPLC) (34, 44) was kindly provided by Martin Low, Columbia College of Physicians and Surgeons, New York, N.Y.

sc$^+$-MNBs. The sc$^+$-MNB cultures were established and grown in minimum essential medium supplemented with 10% fetal bovine serum as described previously (52, 53).

Metabolic labeling of PrP isoforms. Identically seeded, approximately half-confluent 25-cm^2 flasks of sc$^+$-MNBs (52) were preincubated for 6 to 16 h in fresh growth medium, rinsed three times with phosphate-buffered balanced salts solution, and incubated at 37°C in 2 ml of methionine- and cysteine-free minimal essential medium with 1% dialyzed fetal bovine serum. After 60 min, the indicated amount of Tran^{35}S-label (ICN) or Expre^{35}S^{35}S (NEN) was added to each flask for pulse-labeling of designated length. Then 10 ml of chase medium (complete minimal essential medium with 10% fetal bovine serum) was added, and the incubation continued for various chase periods. When the cells were treated with PS, all of the media, starting with the 6- to 16-h preincubation medium (except for the phosphate-buffered balanced salt solution washes) contained 100 ng of PS per ml. Cell lysates and postnuclear supernatants were prepared and methanol precipitated (for the detection of PrP-sen) or treated for the isolation of PrP-res as described below.

Isolation of PrP-res. Cell lysates were prepared, and following the removal of nuclei, debris, and aliquots for the total labeled protein analysis, PrP-res was isolated after proteinase K treatment by ultracentrifugation according to a previously described procedure (15) unless stated otherwise.

Immunoprecipitation of PrP isoforms. The pellets containing metabolically labeled PrP-res or PrP-sen resulting from ultracentrifugation or methanol precipitation were sonicated into a detergent-phospholipid solution and analyzed for labeled PrP-res by immunoprecipitation with a rabbit anti-PrP peptide 89-103 serum (R34) (16), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography as described previously (15) except that Entensify (NEN) was used as the fluor and 20% acrylamide Pharmacia LKB PhastSystem gels were used for SDS-PAGE.

Immunoblotting of PrP-res. Ultracentrifugation pellets resulting from the PrP-res isolation procedure described above were sonicated into 1 to 2 ml of 150 mM NaCl and recentrifuged. The resulting pellets were solubilized by sonication and boiling in 20 to 40 μl of SDS-PAGE sample buffer, separated on a 20% acrylamide gel, and electrophoretically transferred onto an Immobilon-P membrane (Millipore), using the Pharmacia LKB PhastSystem. The membrane was blocked with 5% nonfat dried milk in 10 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.05% Tween 20 (TBST). The filter was incubated for 2 h at ambient temperature with antiserum R34 diluted 1:2,000 in TBST. After being washed in TBST, the filter was incubated for 30 min with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin and developed by using the ECL chemiluminescence detection kit (Amersham). Multiple exposures of each immunoblot were obtained, and estimates of the relative amounts of PrP-res detected in the immunoblot lanes were obtained by comparing the film exposure times giving visually equivalent band intensities.

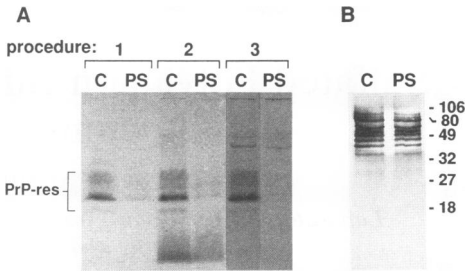

FIG. 1. Effects of PS on the metabolic labeling of PrP-res (A) and total lysate proteins (B). (A) Control sc$^+$-MNB (lanes C) or cells treated with 100 ng of PS per ml (lanes PS) were labeled with [^{35}S]methionine and [^{35}S]cysteine and lysed with detergent as described in Materials and Methods. The PrP-res was distinguished from PrP-sen in the lysates by the standard method of proteinase K treatment followed by the pelleting of PrP-res aggregates by ultracentrifugation as described in Materials and Methods (procedure 1) or the alternative procedures described as follows. Procedure 2 included the proteinase K treatment, but PrP-res was collected by precipitation with 4 volumes of methanol at −20°C instead of ultracentrifugation. In procedure 3, the proteinase K treatment was omitted and PrP-res was pelleted by ultracentrifugation. The resulting pellets were then solubilized and analyzed for PrP-res by immunoprecipitation, SDS-PAGE, and fluorography as described in Materials and Methods. The procedure 3 lanes were from a separate experiment, which accounts for the different background intensity in these lanes. (B) The total lysate proteins were methanol precipitated directly from the detergent lysates of the cells after removal of nuclei and debris by low-speed centrifugation. Equal flask equivalents were loaded onto all lanes in both panels. The positions of molecular mass markers are designated in kilodaltons on the right.

RESULTS

Inhibition of metabolic labeling of PrP-res by PS. One of the most potent polyanions for the chemoprophylaxis of scrapie in vivo is PS (23, 27). To determine whether PS can influence PrP-res metabolism in cells already infected with scrapie, we tested the effect of PS on the accumulation of newly synthesized PrP-res in sc$^+$-MNB cultures. PS (100 ng/ml) greatly reduced the metabolic labeling of PrP-res (Fig. 1A). Since the two generally recognized hallmarks of PrP-res are its protease resistance and its aggregated state, we used a protocol with both a proteinase K treatment and an ultracentrifugation to discriminate PrP-res from normal PrP (procedure 1). To test for the possibility that PS decreased the proteinase K resistance of PrP-res without affecting its aggregation state, or vice versa, samples were also prepared by using only the proteinase K treatment (procedure 2) or the ultracentrifugation (procedure 3) of the cell lysates prior to the solubilization and immunoprecipitation of PrP-res. In each case, the similar PS-dependent reductions in the PrP-res labeling were observed, indicating that PS inhibited the labeling of PrP-res as defined independently by either aggre-

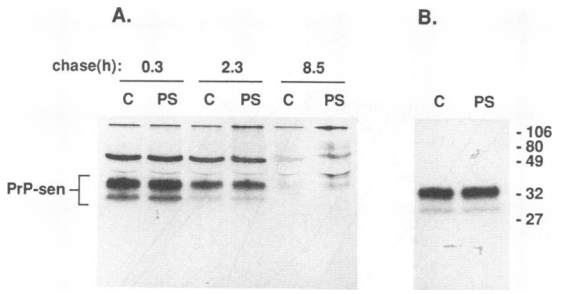

FIG. 2. Lack of effect of PS on the metabolic labeling and turnover of PrP-sen (A) and the transport of PrP-sen to the cell surface (B). (A) Control flasks of sc^+-MNBs (lanes C) were preincubated 16 h and pulse-labeled for 2 h by the general procedure described in Materials and Methods and then incubated in chase medium for the indicated chase time. The cells were then lysed, and methanol precipitates of postnuclear supernatants were analyzed for PrP-sen by immunoprecipitation, SDS-PAGE, and fluorography as described in Materials and Methods. The PS-treated flasks (lanes PS) were treated identically except that the preincubation, pulse, and chase media contained 100 ng of PS per ml. Equal flask equivalents were loaded onto all lanes. (B) PrP-sen was immunoprecipitated from the media of intact sc^+-MNBs treated with PIPLC to release pulse-^{35}S-labeled PrP-sen from the cell surface as described previously (14). Briefly, the cells were preincubated for 16 h, pulse-labeled for 1.5 h, and chased for 0.5 h by the general procedure described in Materials and Methods before treatment with PIPLC for 30 min. PS (100 ng/ml) was included in all media starting with the 16-h preincubation except in the case of the control cells. The positions of molecular mass markers are designated on the left in kilodaltons. Equal flask equivalents were loaded onto all lanes.

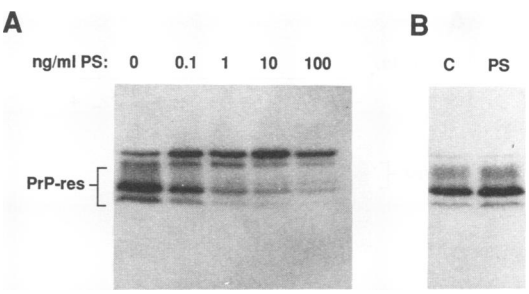

FIG. 3. Inhibition of PrP-res accumulation in cells grown in PS (A) and lack of effect of PS added directly to cell lysate (B). (A) sc^+-MNBs were seeded at a 1:20 dilution of a confluent culture and allowed to attach for 16 h. The designated concentrations of PS were added to the medium, and the cells were allowed to grow for 4 days more to confluence. PrP-res was isolated from proteinase K-treated postnuclear supernatants of the cells by ultracentrifugation and analyzed for PrP by immunoblotting as described in Materials and Methods. (B) Postnuclear supernatant from two 25-cm^2 flask equivalents of sc^+-MNBs was treated with 1 μg of PS per ml (lane PS) or left untreated (lane C), digested with proteinase K, and ultracentrifuged, and the pellet was analyzed by immunoblotting as for panel A. In both panels, each lane represents 0.2 25-cm^2 flask equivalent. The bands designated PrP-res were those whose labeling was specifically eliminated by preabsorption of the antiserum with the synthetic PrP peptide antigen used to generate the antiserum (not shown).

gation or proteinase K resistance. In other experiments, significant but less complete inhibition was also routinely observed at 10 ng of PS per ml (data not shown). The inhibitory effect of PS appeared to be selective for PrP-res because there was no detectable change in the overall profile of labeled proteins in the cell lysates before proteinase K digestion (Fig. 1B). Furthermore, we observed no effects of these concentrations of PS on cellular morphology.

Lack of effect of PS on PrP-sen metabolism. Because phospholipase-sensitive, cell surface PrP-sen is the precursor of PrP-res (15), it was possible that the PS inhibition of PrP-res labeling could be due indirectly to an effect on PrP-sen biosynthesis, turnover, or transport to the cell surface. However, PS at 100 ng/ml had no influence on the metabolic labeling and half-life of PrP-sen (Fig. 2A) or the release of labeled PrP-sen from the cell surface by PIPLC (Fig. 2B). These observations provided evidence that rather than affecting normal PrP metabolism, PS specifically prevented PrP-res formation or greatly reduced its metabolic half-life.

Effect of PS on long-term accumulation of PrP-res. The ability of PS to inhibit the accumulation of newly synthesized PrP-res suggested that, as is the case with Congo red (14), the extended growth of sc^+-MNBs in the presence of PS might reduce the total cellular PrP-res content. Inclusion of concentrations of PS as low as 0.1 ng/ml in the growth medium of 5-day cultures caused substantial reductions in the total PrP-res content detected by immunoblotting with distinct antisera raised against peptides corresponding to PrP residues 89 to 103 (Fig. 3A) or 142 to 155 (data not shown). At 100 ng of PS per ml, the intensity of PrP-res bands in Fig. 3A was ~10% of the control level. Treatment of the cells with PS had no significant negative effect on the growth of the cultures, since the total protein content in the lysates of the PS-treated cells was 100 to 120% of the control level in all cases (data not shown). Furthermore, no changes in cell morphology were observed.

To control for the possibility that residual PS in the lysates artifactually interfered with the immunochemical detection of PrP-res, we added 1 μg of PS per ml to a control cell lysate and found that this treatment did not reduce the PrP-res signal detected by the subsequent PrP-res isolation and immunoblot procedure (Fig. 3B). Thus, without apparent general effects on cellular metabolism, PS greatly reduced the total PrP-res accumulated in the sc^+-MNB cultures.

Long-term effects of transient PS and Congo red treatment. When scrapie-infected cells were grown for 4 days in the presence of PS or Congo red and then passaged three times in control medium, the amount of PrP-res accumulated in the cultures did not recover relative to that in untreated cultures (Fig. 4). Protein assays of the final lysates analyzed for PrP-res indicated that there was no inhibition of cell growth due to the treatment with PS or Congo red that could account for the lower PrP-res content. In addition to the effects of washing and growing the cells in inhibitor-free medium, the three serial passages theoretically diluted any residual cell-associated inhibitor by at least 8,000-fold. Thus, although certain sulfated glycans can remain stably cell associated (28), the serial passages alone would dilute the inhibitors to subinhibitory concentrations in the cultures before the fourth passage. We conclude that single treatments with both Congo red and PS caused long-term reductions in PrP-res content per flask equivalent that persisted beyond the removal of the inhibitor.

Apparent lack of effect of PS and Congo red on existing PrP-res. The inhibition of PrP-res accumulation exhibited by PS and Congo red could be due theoretically to either a reduction in PrP-res formation or a destabilization of PrP-res once it has been formed (14). If the latter were true, it should

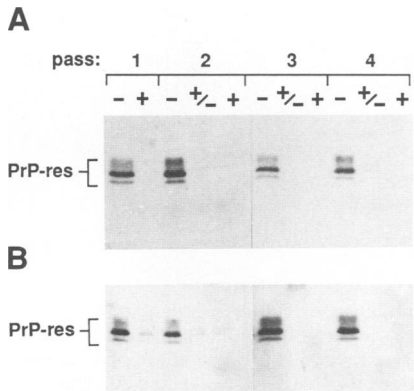

FIG. 4. Lack of recovery of PrP-res levels after removal of Congo red (A) and PS (B). Duplicate 10-cm^2 wells of sc$^+$-MNBs were seeded and grown to confluence in growth medium without inhibitors, with 1 μg of Congo red per ml (A), or with 10 ng of PS per ml (B) as described in the legend to Fig. 3 and Materials and Methods. One well was then serially passaged in the presence or absence of the inhibitor. At each passage, the duplicate wells were lysed and postnuclear supernatants were analyzed for PrP-res by immunoblotting as described in Materials and Methods. Lanes: −, cells never exposed to inhibitor (control); +, cells grown continuously in inhibitor; +/−, cells grown for the first passage in inhibitor and then for all subsequent passages in the absence of inhibitor. Each lane represents 0.2 well equivalent of approximately confluent wells. The variation in intensity of the control PrP-res intensity between passages can be ascribed to differences in cell density at the time of harvest.

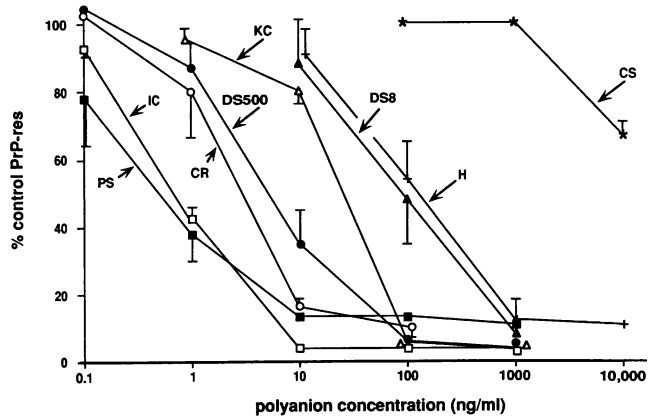

FIG. 6. Relative inhibition of PrP-res accumulation by sulfated glycans and Congo red. Sc$^+$-MNBs were treated with a variety of sulfated glycans and Congo red and analyzed for PrP-res by immunoblotting as described for the PS treatment in the legend to Fig. 3A and Materials and Methods. The PrP-res band intensities were estimated relative to those of untreated controls as described in Materials and Methods and plotted as a function of polyanion concentration. Abbreviations for inhibitors: PS, pentosan polysulfate; IC, iota-carrageenan; CR, Congo red; DS500, dextran sulfate (average MW of 500,000); KC, kappa-carrageenan; DS8, dextran sulfate (average MW of 8,000); H, heparin; CS, chondroitin sulfate (approximately equimolar mixture of types A, B, and C). The data points and error bars represent the averages of two to seven separate determinations ± standard errors of the means.

be possible for PS or Congo red treatment to reduce the total PrP-res accumulated in a sc$^+$-MNB culture to a level below that contained in the cells initially seeded into medium containing the inhibitor. However, as shown in Fig. 5, the minimum PrP-res observed in confluent Congo red- and PS-treated cultures was approximately the same as the input PrP-res in the seeded cells. In some experiments, the PrP-res in Congo red-treated cells appeared to be slightly less than that in the seed cells. These results suggest that PS and Congo red primarily prevented the accumulation of newly synthesized PrP-res and had little, if any, effect on the stability of preexisting PrP-res.

As another test of whether Congo red might directly destabilize existing PrP-res and make it more susceptible to proteolysis, lysates of sc$^+$-MNBs were preincubated for 0 or 23 h with Congo red before being treated with proteinase K and analyzed for PrP-res. Preincubations of PrP-res with as much as 50 μM Congo red (~350 times higher than the minimum concentration found previously to inhibit PrP-res accumulation [14]) had no effect on the PrP-res remaining after proteinase K treatment (data not shown). These results provide additional evidence suggesting that Congo red does not directly influence the susceptibility of preexisting PrP-res to proteolysis.

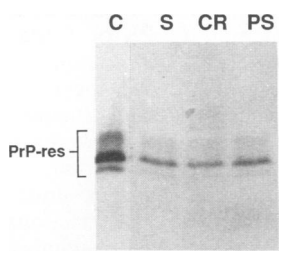

FIG. 5. Comparison of input PrP-res with PrP-res present in PS- and Congo red-treated cells. sc$^+$-MNBs (0.2 25-cm^2 flask equivalent) were seeded into 25-cm^2 flasks in growth medium containing 1 μg of Congo red per ml (lane CR), 100 ng of PS per ml (lane PS), or no inhibitor (lane C) and grown to confluence. The PrP-res content of the seed cells (lane S) was compared with those of the confluent control and inhibitor-treated cells by immunoblotting as described in the legend to Fig. 3 and Materials and Methods. Each lane represents 0.2 confluent 25-cm^2 flask equivalent.

Inhibition of PrP-res accumulation by other sulfated glycans. The anti-PrP-res activities of a variety of other sulfated glycans were compared with those of PS and Congo red. A wide range of potencies in inhibiting the long-term accumulation of PrP-res was observed for sulfated glycans (Fig. 6). Iota-carrageenan, lambda-carrageenan (not shown), and dextran sulfate 500 (average MW of 500,000) were of greatest potency, with substantial inhibition observed at 1 to 10 ng/ml, and were similar in activity to PS and Congo red on a weight/volume basis. Dextran sulfate 8 (average MW of 8,000), kappa-carrageenan, and heparin were of intermediate potency, whereas a mixture of chondroitin sulfates (A, B, and C) was approximately 4 orders of magnitude less inhibitory than PS. In no case was inhibition of cell growth observed at the concentrations tested, since the cultures were similarly confluent at the time of harvest.

Role of sulfates in inhibition of PrP-res accumulation. To test the importance of anionic sulfate groups for the inhibitory properties of a sulfated glycan, we compared the anti-PrP-res activities of dextran sulfate, dextran, and the polycationic DEAE-dextran. The data in Figure 7 indicate that whereas growth of the cells in dextran sulfate 500 inhibited PrP-res accumulation (as shown above), dextran and DEAE-dextran of the same average MW had no activity

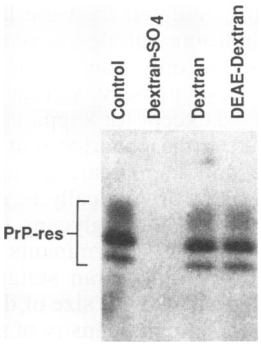

FIG. 7. Comparative effects on PrP-res accumulation of dextran sulfate, dextran, and DEAE-dextran. Sc^+-MNBs were seeded at 1/10 confluent density into medium containing no inhibitor (control) or 1 μg of the designated dextran analog per ml. After growing to a confluent monolayer, the cells were analyzed for PrP-res by immunoblotting as described in the legend to Fig. 3 and Materials and Methods. Each lane represents 0.07 25-cm^2 flask equivalent.

at concentrations that were 10- to 100-fold higher than the MIC of dextran sulfate (according to the data in Fig. 6). These results indicate that the presence of negatively charged sulfates play an important role in the inhibition by dextran sulfate.

Lack of inhibition by carboxylate and phosphate polyanions and amphotericin B. In experiments similar to those shown in Fig. 6 and 7, we found that other organic polyanionic polymers with carboxylates or phosphates instead of sulfates, i.e., polygalacturonic acid, polyglutamic acid, salmon sperm DNA (sonicated), and yeast tRNA, did not inhibit the accumulation of PrP-res in the concentration range tested (≤10 μg/ml; not shown). This provided additional evidence that not all polyanionic glycans or polymers are inhibitors of PrP-res accumulation.

The fungicide amphotericin B was also tested for inhibition of PrP-res accumulation because it has recently been shown to delay the accumulation of PrP-res in hamsters infected with the 263K strain of the scrapie agent but not in scrapie agent-infected mice (63). As might be anticipated from the in vivo results, amphotericin B (≤10 μg/ml) had no effect on PrP-res accumulation in our scrapie agent-infected mouse cells (data not shown).

DISCUSSION

Possible therapeutic antiscrapie mechanism of sulfated polyanions in vivo. Previous studies of the prophylactic effects of polyanions on scrapie in vivo have suggested that these compounds may be useful in the prevention of a variety of TSEs, especially if high-risk individuals are identified before the onset of disease (23, 27, 40, 41, 49). Such antiscrapie drugs may also be helpful in dissecting the mechanism of agent replication and pathogenesis. Unfortunately, the mechanistic interpretation of their effects in vivo is complicated by the fact that there are many activities of sulfated polyanions which might influence the pathogenesis of the ill-defined scrapie agent. These include the stimulation of immune responses (9, 21, 26, 45, 60) and the inhibition of viral functions (3, 4, 47, 59, 62) and of phagosome-lysosome fusion in macrophages (6, 33). However, in this study, we have demonstrated that sulfated polyanions can act directly on infected cells derived from a likely clinical target, i.e., neurons, to inhibit the accumulation of PrP-res. Given these results and the apparent importance of PrP-res in pathogenesis, it is possible that the therapeutic activity of these compounds is due specifically to the inhibition of PrP-res accumulation. This may also be the case for the nonpolyanionic drug amphotericin B (63).

As a test of this possibility for the polyanions, we compared the relative antiscrapie activities of various sulfated polyanions in vivo with their relative potencies as inhibitors of PrP-res accumulation in vitro. PS, lambda-carrageenan, and dextran sulfate 500 have the greatest therapeutic potencies in vivo, whereas heparin and chondroitin sulfate have little or no therapeutic effect (23, 27–29, 41, 43). We observed a similar relationship in vitro, with PS, lambda-carrageenan, iota-carrageenan, and dextran sulfate 500 being orders of magnitude more potent as inhibitors of PrP-res accumulation than heparin or chondroitin sulfate. Thus, there is a correspondence between the in vivo therapeutic and in vitro anti-PrP-res activities of the sulfated glycans tested. This finding is consistent with the hypothesis that at least one prophylactic mechanism of the sulfated glycans involves inhibition of PrP-res accumulation.

If sulfated glycans can exert potent anti-PrP-res activity in neuron-derived cells already infected with scrapie, as we have shown in vitro, it is somewhat puzzling that these inhibitors are so ineffective in vivo when administered after the infection has reached the central nervous system but before massive, presumably pathogenic accumulation of PrP-res in this tissue. One possible explanation could be that the blood-brain barrier protects infected brain cells from these inhibitors. Alternatively, the sc^+-MNBs may, for some unknown reason, be a poor model of scrapie-infected cells of the central nervous system in this respect. Further studies will be required to clearly discriminate between these and other possibilities.

Potential mechanisms of inhibition of PrP-res accumulation. Although the mechanism of action of Congo red and sulfated glycans remains uncertain, the potent inhibition exhibited by these compounds should make them useful tools for elucidating the cellular and molecular mechanisms of PrP-res accumulation. Insights gained from studies of PrP-res may be applicable to other systems in which abnormally stable, potentially amyloidogenic proteins are made. A pertinent feature of all amyloid plaques, including those composed of PrP-res (57, 58), is the presence of highly sulfated glycosaminoglycans (GAGs) (reviewed in reference 48). This observation led to a suggestion that these endogenous sulfated proteoglycans, specifically heparan sulfate proteoglycans, may be involved in the polymerization of proteins into amyloid filaments. Our present demonstration that exogenous sulfated glycans and Congo red can interfere with PrP-res accumulation is consistent with the idea that endogenous GAGs play a functional role in amyloidogenesis. More specifically, we speculate that these inhibitors bind to PrP-res or PrP-sen and competitively inhibit an interaction with a specific cellular sulfated GAG that is essential for PrP-res formation or stabilization. Implicit in this hypothesis is the idea that the inhibitors can bind PrP but lack certain features required to facilitate PrP-res accumulation within cells by themselves.

Endogenous sulfated GAGs are commonly found on the cell surface or in the extracellular matrix (56), and PrP-sen is exposed on the cell surface before it is converted to PrP-res (15). Therefore, exogenous, membrane-impermeant inhibitors should have access to PrP-sen at the site where an interaction with endogenous GAGs would likely occur before or during the formation of PrP-res. We have not yet

demonstrated that any of sulfated glycan inhibitors can actually bind PrP, but others have shown that Congo red binds to amyloid fibrils of PrP-res (51). The binding of sulfated GAGs to polypeptides can affect protein folding and fibril formation and induce conformational shifts from α-helix to the β-sheet structure that is predominant in amyloid fibrils (reviewed in reference 42), including those comprising PrP-res (17). If endogenous sulfated GAGs play an essential role in PrP-res accumulation, it is conceivable that differential GAG expression influences whether or not a given cell can accumulate PrP-res and thus be a clinical target in TSEs. Furthermore, GAG expression and metabolism may be altered by TSE infections to affect disease pathogenesis.

Other indirect mechanisms for the inhibition of PrP-res accumulation by Congo red and sulfated glycans can also be envisioned. For instance, PrP-res may not be a necessary component of the transmissible scrapie agent and therefore may be produced as a pathogenic by-product of the infection (19, 63). In this scenario, Congo red and/or the sulfated glycans could inhibit PrP-res accumulation by affecting the scrapie agent rather than PrP-res accumulation directly. As noted earlier, these compounds inhibit a number of functions of conventional viruses. In addition, since polyanions have been known to affect membrane trafficking events in phagocytic cells, such as phagosome-lysosome fusion (6, 33), it is conceivable that rather than interacting with PrP directly, the sulfated polyanions influence subcellular translocations of PrP that may be important for PrP-res formation or longevity.

When evaluating the relationship of PrP-res to the scrapie agent, it is interesting to consider the possible implications of the observation that the rate of PrP-res accumulation in vitro did not recover to control levels after Congo red and PS were removed from the medium and diluted to subactive levels within the cells by serial passage (Fig. 4). If PrP-res were not a component of the scrapie agent and its accumulation were secondary to scrapie agent replication, then direct inhibition of PrP-res accumulation by Congo red and PS would not be expected, a priori, to reduce the amount of agent in the cells. In this case, the agent would still be present after treatment to reinitiate the formation of PrP-res. However, because the rate of PrP-res accumulation did not recover, it appears more likely that the depletion of PrP-res by Congo red and PS also depleted the transmissible agent. A definitive answer on this point will require the long-term scrapie agent bioassay of treated cell extracts in mice (in progress).

Although amphotericin B inhibits PrP-res accumulation in one (and only one) model of scrapie in hamsters (63), the fact that it is not polyanionic and has no effect on PrP-res accumulation in our in vitro system suggests that it operates by a strain- and species-specific mechanism that differs from that of the sulfated polyanions or Congo red.

The molecular attributes of effective PrP-res inhibitors. The importance of sulfates for the inhibition of PrP-res accumulation was clearly demonstrated by the comparison of dextran sulfate, dextran, and DEAE-dextran (Fig. 8). Although the nonsulfated polyanions that we tested were not effective as inhibitors, it remains possible that other types of nonsulfated polyanions are inhibitory. For instance, one nonsulfate antiviral polyanion, heteropolyanion 23 (ammonium 5-tungsto-2-antimoniate), has potent prophylactic antiscrapie activity in vivo (40) and might operate by the anti-PrP-res mechanism that we have suggested for the sulfated glycan inhibitors. The difference in potency between iota- and kappa-carrageenan suggests that the degree of sulfation of glycans also affects their inhibitory properties because the former is more highly sulfated than the latter (2). The low potency of the chondroitin sulfates is another indication of this, since they contain only one sulfate per disaccharide monomer and are less densely sulfated than the other sulfated glycans tested except for kappa-carrageenan, which also has one sulfate per disaccharide unit (2). The fact that chondroitin sulfate and kappa-carrageenan are similar in sulfate density but differ dramatically in inhibitory activity indicates that properties of the glycans other than sulfate density are also important determinants of potency. The slightly greater potency of dextran sulfate 500 relative to dextran sulfate 8 suggests that the size of the glycan polymer is another factor. Interestingly, density of sulfate groups and glycan size usually correlate with the efficiency of binding of natural sulfated GAGs with their ligands (reviewed in reference 56). Other properties of sulfated glycans that might be expected to influence PrP-res inhibition include the positioning of sulfates, the presence of other nonsulfate substituents, the properties of the glycan backbone (e.g., flexibility and conformation), associations between glycan strands (e.g., double-helix formation by the carrageenans [2]), and the half-life of the glycan in the cell culture.

Although Congo red is sulfonated, it is much smaller than the sulfated glycans tested and contains no carbohydrate. Therefore, we cannot be sure whether it has precisely the same mechanism of action as do the sulfated glycans or whether the sulfonates are important for its activity. Additional studies with analogs of Congo red and sulfated glycans will be required to further refine our understanding of the qualities of PrP-res inhibitors and the cellular mechanism(s) of PrP-res formation.

ACKNOWLEDGMENTS

We thank Richard Race for providing the scrapie agent-infected neuroblastoma cells, and we thank Bob Evans and Gary Hettrick for assistance with graphics.

REFERENCES

1. **Aiken, J. M., and R. F. Marsh.** 1990. The search for scrapie agent nucleic acid. Microbiol. Rev. **54**:242–246.
2. **Anderson, N. S., J. W. Campbell, M. M. Harding, D. A. Rees, and J. W. B. Samuel.** 1969. X-ray diffraction studies of polysaccharide sulphates: double helix models for kappa- and iota-carrageenans. J. Mol. Biol. **45**:85–99.
3. **Baba, M., M. Nakajima, D. Schols, R. Pauwels, J. Balzarini, and E. DeClercq.** 1988. Pentosanpolysulfate, a sulfated oligosaccharide, is potent and selective anti-HIV agent in vitro. Antiviral Res. **9**:335–349.
4. **Beisert, L., H. Suhartono, I. Winkler, C. Meinchsner, H.-D. Helsberg, E. Schrinner, H.-D. Brede, and H. Rubsamen-Waigmann.** 1988. Inhibition of HIV replication of polysulfated polyxylan: HOE/Bay 946, a novel antiviral compound. AIDS **2**:449–457.
5. **Bendheim, P. E., R. A. Barry, S. J. DeArmond, D. P. Stites, and S. B. Prusiner.** 1984. Antibodies to a scrapie prion protein. Nature (London) **310**:418–421.
6. **Bloksma, N., M. J. DeReuver, and J. M. N. Willers.** 1980. Influence on macrophage functions as a possible basis of immunomodification by polyanions. Ann. Immunol. **131D**:255–265.
7. **Bolton, D. C., M. P. McKinley, and S. B. Prusiner.** 1982. Identification of a protein that purifies with the scrapie prion. Science **218**:1309–1311.
8. **Borchelt, D. R., M. Scott, A. Taraboulos, N. Stahl, and S. B. Prusiner.** 1990. Scrapie and cellular prion proteins differ in the kinetics of synthesis and topology in cultured cells. J. Cell Biol. **110**:743–752.
9. **Bradfield, J. W. B., and G. V. R. Born.** 1974. Lymphocytosis induced by heparin and other sulphated polysaccharides in mice and rats. Cell. Immunol. **14**:22–32.

10. **Brown, P., M. Coker-Vann, K. Pomeroy, M. Franko, D. M. Asher, C. J. Gibbs, Jr., and D. C. Gajdusek.** 1986. Diagnosis of Creutzfeldt-Jakob disease by Western blot identification of marker protein in human brain tissue. N. Engl. J. Med. **314:**547–551.
11. **Bruce, M. E., P. A. McBride, and C. F. Farquhar.** 1989. Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie. Neurosci. Lett. **102:** 1–6.
12. **Carlson, G. A., D. T. Kingsbury, P. A. Goodman, S. Coleman, S. T. Marshall, S. DeArmond, D. Westaway, and S. B. Prusiner.** 1986. Linkage of prion protein and scrapie incubation time genes. Cell **46:**503–511.
13. **Caughey, B.** 1991. Cellular metabolism of normal and scrapie-associated forms of PrP. Semin. Virol. **2:**189–196.
14. **Caughey, B., and R. E. Race.** 1992. Potent inhibition of scrapie-associated PrP accumulation by Congo red. J. Neurochem. **59:**768–771.
15. **Caughey, B., and G. J. Raymond.** 1991. The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J. Biol. Chem. **266:** 18217–18223.
16. **Caughey, B., G. J. Raymond, D. Ernst, and R. E. Race.** 1991. N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. J. Virol. **65:**6597–6603.
17. **Caughey, B. W., A. Dong, K. S. Bhat, D. Ernst, S. F. Hayes, and W. S. Caughey.** 1991. Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy. Biochemistry **30:**7672–7680.
18. **Chesebro, B., R. Race, K. Wehrly, J. Nishio, M. Bloom, D. Lechner, S. Bergstrom, K. Robbins, L. Mayer, J. M. Keith, C. Garon, and A. Haase.** 1985. Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. Nature (London) **315:**331–333.
19. **Czub, M., H. R. Braig, and H. Diringer.** 1988. Replication of the scrapie agent in hamsters infected intracerebrally confirms the pathogenesis of an amyloid-inducing virosis. J. Gen. Virol. **69:**1753–1756.
20. **DeArmond, S. J., W. C. Mobley, D. L. DeMott, R. A. Barry, J. H. Beckstead, and S. B. Prusiner.** 1987. Changes in the localization of brain prion proteins during scrapie infection. Neurology **37:**1271–1280.
21. **Diamantstein, T., B. Wagner, I. Beyso, M. V. Odenwald, and G. Schultz.** 1971. Stimulation of humoral antibody formation by polyanions. II. The influence of sulfate esters of polymers on the immune response in mice. Eur. J. Immunol. **1:**340–343.
22. **Diedrich, J. F., P. E. Bendheim, Y. S. Kim, R. I. Carp, and A. T. Haase.** 1991. Scrapie-associated prion protein accumulates in astrocytes during scrapie infection. Proc. Natl. Acad. Sci. USA **88:**375–379.
23. **Diringer, H., and B. Ehlers.** 1991. Chemoprophylaxis of scrapie in mice. J. Gen. Virol. **72:**457–460.
24. **Diringer, H., H. Gelderblom, H. Hilmert, M. Ozel, C. Edelbluth, and R. H. Kimberlin.** 1983. Scrapie infectivity, fibrils and low molecular weight protein. Nature (London) **306:**476–478.
25. **Doh-ura, K., J. Tateishi, H. Sasaki, T. Kitamoto, and Y. Sakaki.** 1989. Pro→Leu change at position 102 of prion protein is the most common but not the sole mutation related to Gerstmann-Straussler syndrome. Biochem. Biophys. Res. Commun. **163:** 974–979.
26. **Dorries, R., A. Schimpl, and E. Wecker.** 1974. Action of dextran sulfate as a direct and general B-cell mitogen. Eur. J. Immunol. **4:**230–233.
27. **Ehlers, B., and H. Diringer.** 1984. Dextran sulphate 500 delays and prevents mouse scrapie by impairment of agent replication in spleen. J. Gen. Virol. **65:**1325–1330.
28. **Ehlers, B., R. Rudolf, and H. Diringer.** 1984. The reticuloendothelial system in scrapie pathogenesis. J. Gen. Virol. **65:**423–428.
29. **Farquhar, C. F., and A. G. Dickinson.** 1986. Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. J. Gen. Virol. **67:**463–473.
30. **Glenner, G. G.** 1980. Amyloid deposits and amyloidosis: the beta-fibrillosa (second of two parts). N. Engl. J. Med. **302:**1333–1343.
31. **Goldgaber, D., L. G. Goldfarb, P. Brown, D. M. Asher, W. T. Brown, S. Lin, J. W. Teener, S. M. Feinstone, R. Rubenstein, R. J. Kascsak, et al.** 1989. Mutations in familial Creutzfeldt-Jakob disease and Gerstmann-Straussler-Scheinker's syndrome. Exp. Neurol. **106:**204–206.
32. **Goldmann, W., N. Hunter, J. D. Foster, J. M. Salbaum, K. Beyreuther, and J. Hope.** 1990. Two alleles of a neural protein gene linked to scrapie in sheep. Proc. Natl. Acad. Sci. USA **87:**2476–2480.
33. **Hart, P. D., and M. R. Young.** 1975. Interference with normal phagosome-lysosome fusion in macrophages, using ingested yeast cells and suramin. Nature (London) **256:**47–49.
34. **Henner, D. J., M. Yang, E. Chen, R. Hellmiss, H. Rodriguez, and M. G. Low.** 1988. Sequence of the Bacillus thuringiensis phosphatidylinositol specific phospholipase C. Nucleic Acids Res. **16:**10383.
35. **Hope, J., L. J. D. Reekie, N. Hunter, G. Multhaup, K. Beyreuther, H. White, A. C. Scott, M. J. Stack, M. Dawson, and G. A. H. Wells.** 1988. Fibrils from brains of cows with new cattle disease contain scrapie-associated protein. Nature (London) **336:**390–392.
36. **Hsiao, K., H. F. Baker, T. J. Crow, M. Poulter, F. Owen, J. D. Terwilliger, D. Westaway, J. Ott, and S. B. Prusiner.** 1989. Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. Nature (London) **338:**342–345.
37. **Hsiao, K. K., M. Scott, D. Foster, D. F. Groth, S. J. DeArmond, and S. B. Prusiner.** 1990. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science **250:**1587–1590.
38. **Hunter, N., J. Hope, I. McConnell, and A. G. Dickinson.** 1987. Linkage of the scrapie-associated fibril protein (PrP) gene and Sinc using congenic mice and restriction fragment length polymorphism analysis. J. Gen. Virol. **68:**2711–2716.
39. **Kimberlin, R. H.** 1990. Scrapie and possible relationships with viroids. Semin. Virol. **1:**153–162.
40. **Kimberlin, R. H., and C. A. Walker.** 1983. The antiviral compound HPA-23 can prevent scrapie when administered at the time of infection. Arch. Virol. **78:**9–18.
41. **Kimberlin, R. H., and C. A. Walker.** 1986. Suppression of scrapie infection in mice by heteropolyanion 23, dextran sulfate, and some other polyanions. Antimicrob. Agents Chemother. **30:**409–413.
42. **Kisilevsky, R.** 1987. From arthritis to Alzheimer's disease: current concepts on the pathogenesis of amyloidosis. Can. J. Physiol. Pharmacol. **65:**1805–1815.
43. **Ladogana, A., P. Casaccia, L. Ingrosso, M. Cibati, M. Salvatore, Y. G. Xi, C. Masullo, and M. Pocchiari.** 1992. Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. J. Gen. Virol. **73:**661–665.
44. **Low, M. G., J. Stiernberg, G. L. Waneck, R. A. Flavell, and P. W. Kincade.** 1988. Cell-specific heterogeneity in sensitivity of phosphatidylinositol-anchored membrane antigens to release by phospholipase C. J. Immunol. Methods **113:**101–111.
45. **McCarthy, R. E., L. W. Arnold, and G. F. Babcock.** 1977. Dextran sulfate: an adjuvant for cell-mediated immune responses. Immunology **32:**963–974.
46. **McKinley, M. P., A. Taraboulos, L. Kenaga, D. Serban, A. Stieber, S. J. DeArmond, S. B. Prusiner, and N. Gonatas.** 1991. Ultrastructural localization of scrapie prion proteins in cytoplasmic vesicles of infected cultured cells. Lab. Invest. **65:**622–630.
47. **Moelling, K., T. Schulze, and H. Diringer.** 1989. Inhibition of human immunodeficiency virus type 1 RNase H by sulfated polyanions. J. Virol. **63:**5489–5491.
48. **Narindrasorasak, S., D. Lowery, P. Gonzalez-DeWhitt, R. A. Poorman, B. Greenberg, and R. Kisilevsky.** 1991. High affinity interactions between the Alzheimer's beta-amyloid precursor proteins and the basement membrane form of heparan sulfate proteoglycan. J. Biol. Chem. **266:**12878–12883.

49. **Pocchiari, M., M. Salvatore, A. Ladogana, L. Ingrosso, Y. G. Xi, M. Cibati, and C. Masullo.** 1991. Experimental drug treatment of scrapie: a pathogenetic basis for rationale therapeutics. Eur. J. Epidemiol. **7**:556–561.
50. **Prusiner, S. B.** 1991. Molecular biology of prion diseases. Science **252**:1515–1522.
51. **Prusiner, S. B., M. P. McKinley, K. A. Bowman, P. E. Bendheim, D. C. Bolton, D. F. Groth, and G. G. Glenner.** 1983. Scrapie prions aggregate to form amyloid-like birefringent rods. Cell **35**:349–358.
52. **Race, R. E., B. Caughey, K. Graham, D. Ernst, and B. Chesebro.** 1988. Analyses of frequency of infection, specific infectivity, and prion protein biosynthesis in scrapie-infected neuroblastoma cell clones. J. Virol. **62**:2845–2849.
53. **Race, R. E., L. H. Fadness, and B. Chesebro.** 1987. Characterization of scrapie infection in mouse neuroblastoma cells. J. Gen. Virol. **68**:1391–1399.
54. **Race, R. E., K. Graham, D. Ernst, B. Caughey, and B. Chesebro.** 1990. Analysis of linkage between scrapie incubation period and the prion protein gene in mice. J. Gen. Virol. **71**:493–497.
55. **Rohwer, R. G.** 1991. The scrapie agent: "a virus by any other name." Curr. Top. Microbiol. Immunol. **172**:195–232.
56. **Ruoslahti, E.** 1988. Structure and biology of proteoglycans. Annu. Rev. Cell Biol. **4**:229–255.
57. **Snow, A. D., R. Kisilevsky, J. Willmer, S. B. Prusiner, and S. J. DeArmond.** 1989. Sulfated glycosaminoglycans in amyloid plaques of prion diseases. Acta Neuropathol. **77**:337–342.
58. **Snow, A. D., T. N. Wight, D. Nochlin, Y. Koike, K. Kimata, S. J. DeArmond, and S. B. Prusiner.** 1990. Immunolocalization of heparan sulfate proteoglycans to the prion protein amyloid plaques of Gerstmann-Straussler syndrome, Creuzfeldt-Jakob disease and scrapie. Lab. Invest. **63**:601–611.
59. **Takemoto, K. K., and P. Fabisch.** 1964. Inhibition of herpes virus by natural and synthetic acid polysaccharides. Proc. Soc. Exp. Biol. Med. **116**:140–144.
60. **Vogt, W., H. Ruhl, B. Wagner, and T. Diamantstein.** 1973. Stimulation of DNA synthesis in mouse lymphoid cells by polyanions in vitro. II. Relationship between adjuvant activity and stimulation of DNA synthesis by polyanions. Eur. J. Immunol. **3**:493–496.
61. **Woody, A. M., R. R. Reisbig, and R. W. Woody.** 1981. Spectroscopic studies of Congo red binding to RNA polymerase. Biochim. Biophys. Acta **655**:82–88.
62. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63**:52–58.
63. **Xi, Y. G., L. Ingrosso, A. Ladogana, C. Masullo, and M. Pocchiari.** 1992. Amphotericin B treatment dissociates in vivo replication of the scrapie agent from PrP accumulation. Nature (London) **356**:598–601.