Use of streptomycin for precipitation and detection of proteinase K resistant prion protein (PrP^{sc})

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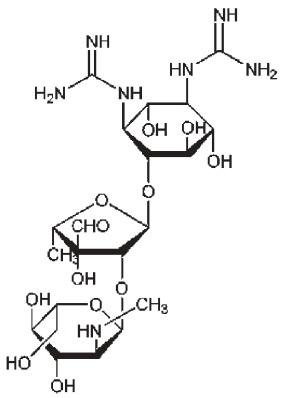
The ability of streptomycin to form multimolecular aggregates with pathogenic prion proteins and their recovery by precipitation *via* a low-speed centrifugation step has been demonstrated; these novel properties of streptomycin make it a useful substance that increases the sensitivity of laboratory diagnostic techniques for prion infections in man and animals

Conformational changes in the cellular glycoprotein PrP^{c} can lead to propagation of a β-sheet conformed, detergent insoluble and proteinase-K (PK) resistant pathogenic protein, PrP^{sc}.[1,2] The accumulation of this PrP^{sc} in animals is associated with a wide range of transmissible neurodegenerative spongiform encephalopathies (TSE), including scrapie in sheep, spongiform encephalopathy in cattle (BSE), chronic wasting disease in deer and Creuztfeldt–Jakob disease in humans.[3]

The antigenic PrP^{sc} load is generally thought to increase progressively after infection, in lymphoid and peripheral nervous tissue and to reach the brain where its aggregation determines the fatal neuropathological issue of TSEs. Thus, apart from the brain in which PrP^{sc} accumulation is a dead-end, PrP^{sc} concentration is expected to be low in other tissues or in biological samples such as blood. The recent discovery of a new variant of Creuztfeldt–Jakob disease transmissible by blood transfusion requires tests that allow detection of PrP^{sc} at very low concentrations in biological fluids.

We have thus been interested in screening molecules for their ability to either capture and concentrate, or precipitate PrP^{sc}. In this paper we present results obtained with one of the selected molecules, streptomycin (Scheme 1), a well-known antibiotic which was revealed to be capable of aggregating PrP^{sc}. The work was further extended to functionally equivalent molecules.

Initial work involved screening of a series of antibiotics, Fig. 1, of these only streptomycin showed an increase in the molecular weight indicating complexation. Streptomycin was thus used to create appropriate conditions for concentrating and separating the PrP^{sc} PK-resistant fragment PrP^{res} by flocculation and precipitation. The addition of increasing quantities of streptomycin sulfate to constant amounts of the non-soluble fraction of PrP^{sc} extracted from sheep or mouse brains,[4] followed by electrophoresis on 15% polyacrylamide gel, transfer and immunodetection revealed an increase in the apparent molecular mass of each of the three PrP^{sc} bands. These arise from the non-glycosylated form of PrP (molecular mass of about 19 kDa for bovine PrP or about 21 kDa for ovine PrP) and the mono- and bi-glycosylated glycoforms (having molecular masses of about 25 kDa and about 28 kDa, respectively).[5] The prion molecular mass increase for the three bands was proportional to the quantity of added streptomycin. At lower quantities of streptomycin the non-glycosylated protein band was the first to show an increase in its apparent molecular mass and was progressively followed by the mono-glycosylated band and finally the bi-glycosylated band as the streptomycin quantities increased (Fig. 2(a) and (b)). It can be



Scheme 1 Molecular structure of streptomycin.

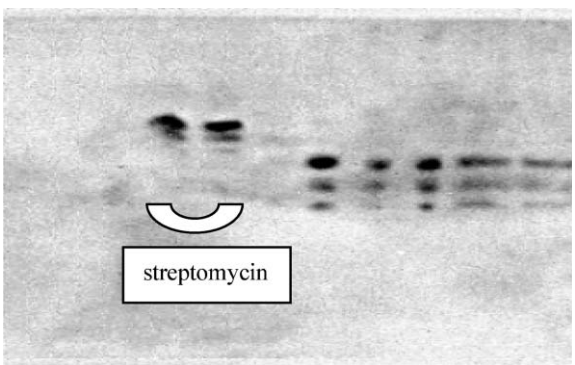


Fig. 1 Blots of PrP^{res} extracted from ovine brains and treated with streptomycin sulfate at concentrations of 120 mM and 60 mM, lanes 1 and 2 respectively, and treated with penicillin G sodium salt, polymyxin B sulfate, geneticin (G418), ampicillin and amphotericin B, lanes 3–7.†

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estimated that 10–12 streptomycin molecules per PrP monomer were required to be complexed so as to account for the increase of the molecular mass of each PrP^{sc} band.

Further analysis of molecules sharing common chemical functions with streptomycin and susceptible to reproduce its effects on PrP^{sc}, provided evidence that they should at least contain two guanidinium and/or ammonium groups. That dihydrostreptomycin in which the aldehyde group is reduced gives identical results ruled out the possibility of a Schiff-base reaction participating in the aggregation process. An example of such functional equivalence is shown for bis-3-aminopropylamine in Fig. 3. Increasing apparent molecular weight of the prion proteins is proportional to the concentration of these aggregating molecules, whereas the three bands tend to migrate altogether in a smeared distribution of aggregated proteins.

When higher concentrations of streptomycin sulfate were used with PK-digested brain homogenates from scrapie infected sheep, it caused aggregation and flocculation of PK-resistant PrP^{sc} fragments (PrP^{res}) with gradual recovery in the precipitate after low-speed centrifugation. Complete PrP^{res} precipitation was achieved by the addition of 20% v/v of a 0.7 M streptomycin solution, Fig. 4(a). Streptomycin was further found to precipitate cattle, mouse and human PrP^{res}. Similarly dihydrostreptomycin sesquisulfate allowed total precipitation of PrP^{sc} under the same conditions (Fig. 4(b)).

We further observed that addition of streptomycin increased the sensitivity of PrP^{res} detection in serially diluted bovine BSE brain samples. The results revealed positive detection at the dilutions 1/16 in the absence and at dilutions of 1/64 and 1/256 when 5 and 10 μL of a 0.7 M solution of streptomycin were added (Fig. 5).

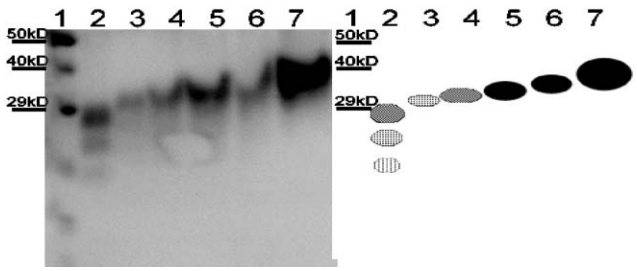

Fig. 3 Western blot of 5 μl of the non-soluble fraction of PrP^{SC}, purified from 1.5 mg of brain tissue from scrapie infected sheep per tube and concentrations of 0, 30, 60, 120, 180 and 240 mM of bis-3-aminopropylamine, respectively, from lane 2 to 7, and molecular weight marker lane 1.

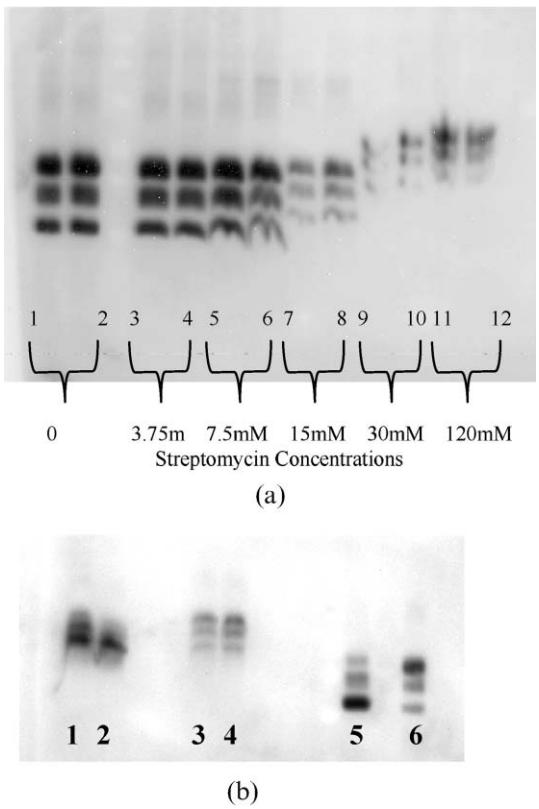

Fig. 2 (a) Western blot: 10 μl of the non-soluble fraction of PrP^{sc} extracted from 2 mg of scrapie infected sheep brain tissue per tube, and concentrations of 0 (lanes 1 and 2), 3.75 (lanes 3 and 4), 7.5 (lanes 5 and 6), 15 (lanes 7 and 8), 30 (lanes 9 and 10) and 120 mM (lanes 11 and 12) of streptomycin. (b) Western blot of mixtures of the non-soluble fraction of PrP^{sc} extracted from 5 mg of scrapie infected sheep brain partially deglycosylated with PNGase[6] (lanes 1, 2 and 5) or BSE infected mouse lanes (3, 4 and 6) to which was added 0 (lanes 5 and 6), 1 μL of a 0.7 M solution (lanes 2 and 4) and 2 μL of a 0.7 M solution of streptomycin (lanes 1 and 3).

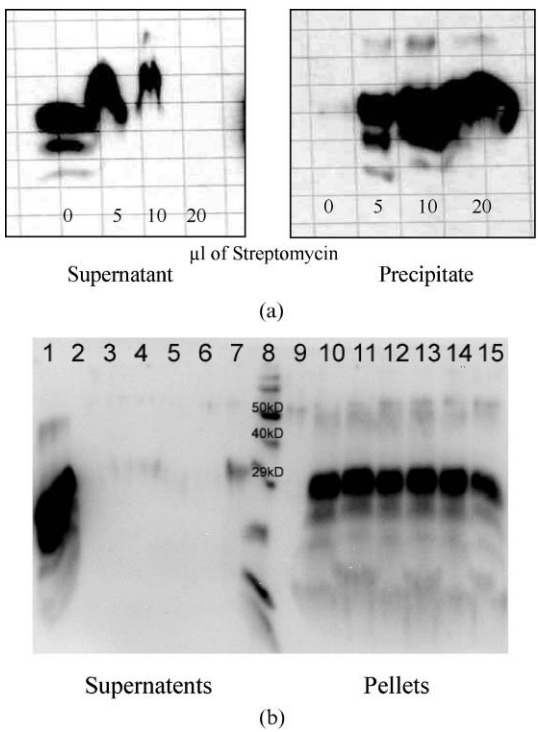

Fig. 4 (a) Precipitation of the non-soluble fraction of PrP^{res} by streptomycin; 10% homogenates in 5% glucose from scrapie infected sheep (equivalent to 10 mg of brain tissue) were treated with proteinase K and to 100 μl was added 0, 5, 10 or 20 μl of streptomycin at 0.7 M. (b) Precipitation of the non-soluble fraction of PrPres (lanes 1–7 supernatants and 9–15 pellets). Proteinase K treated 10% brain homogenates (equivalent 10 mg brain tissue) were incubated with 0, 2.5, 5 or 10 μl of streptomycin at 0.7 M or 2.5, 5 or 10 μl dihydrostreptomycin sesquisulfate at 0.7 M.‡ Lanes 1 and 9 without additive, lanes 2–4 and 10–12 with streptomycin, lanes 5–7 and 13–15 with dihydrostreptomycin sesquisulfate and molecular weight marker lane 8.

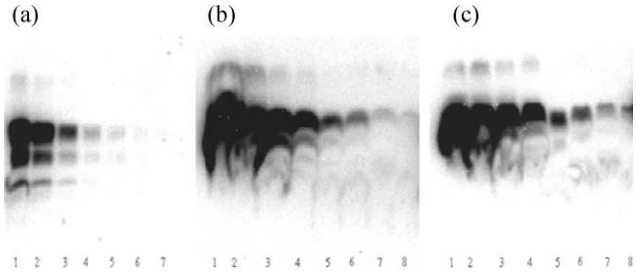

Fig. 5 The use of streptomycin sulfate in the detection of PrP^{sc} in a BSE brain homogenate was demonstrated following a serial 1/2 dilution in 5% glucose (three sets). 1 μg of PK was added to 100 μl of homogenate in each tube of the three sets with 0, 5, 10 μl of streptomycin at 0.7 M added to set 1, 2 and 3, respectively.§ (a) Serial dilutions from 1/1 to 1/64, without streptomycin. (b) Serial dilutions from 1/2 to 1/256 with 5 μL streptomycin (0.7 M). (c) Serial dilutions from 1/2 to 1/256 with 10 μL streptomycin (0.7 M).

The mechanism of interaction with streptomycin as well as the other substances possessing two guanidine groups and/or two ammonium functions with the prion proteins is most likely to occur *via* electrostatic interaction between each of these chemical groups and different amino acids from the same or from different PrP molecules. When different PrP molecules or fragments are cross-linked by such a proportionally very small molecule, reticulation is expected, thus leading to the formation of flocculated aggregates in liquid solutions. Hence, streptomycin and other functional equivalents bind efficiently to the non-glycosylated and both glycoforms of PrP.

Streptomycin is generally added as an antibiotic into the cell culture media. The capability of streptomycin to bind to prion proteins as shown here has to be taken in consideration whenever recombinant prion protein are produced in eukaryote cells,[7,8] insect cell cultures or in PrP^{res} infected cell cultures[9–11] as streptomycin is often used in the production of such proteins. Whether the infectivity of PrPres is reduced, equivalent or enhanced after reticulation by streptomycin remains to be verified by mice inoculation.

Many substances have been used for the concentration of the prion protein: detergents such as Sarkosyl salts help precipitation of insoluble PrP^{sc} by ultra-centrifugation,[12] monoclonal antibodies can be used for the immuno-precipitation of the PrP^{sc} protein,[13] sodium phosphotungstic acid (PTA)[14] and trichloroacetic acid (TCA)[15] have also been used and lastly inorganic polyanions such as polyoxometalate also precipitated PrP^{sc}.[16] Nonetheless, the features of aggregation and flocculation of PrP^{res} by streptomycin and its functional equivalents, which thus allow precipitation and concentration of PrP by low-speed centrifugation or simple sedimentation, are highly original.

In conclusion, the addition of increasing volumes of streptomycin to a tissue homogenate containing PrP^{sc} or PrP^{res}, when digested by proteinase K, induced the formation of multimolecular aggregates of streptomycin–PrP molecules and their recovery by precipitation *via* a low-speed centrifugation step. The immunological reactivity of PrP^{res} with specific antibodies in presence of attached streptomycin molecules was not altered. These novel properties of streptomycin, to precipitate the pathogenic prion proteins, make it a useful substance that increases the sensitivity of the employed laboratory diagnostic techniques for prion infections in humans and animals. This was shown here with western blots, but we are now further evaluating streptomycin for concentrating and extracting PrP^{res} from biological fluids, thus allowing its detection in classical immunoassay methods and, in particular, by ELISA microplate techniques.

Notes and references

† The antibiotics used were each mixed with 2.4 mg of brain tissue equivalent extracted ovine PrP^{res} in 10 μl volume in laemmli electrophoresis buffer.[17] The mixtures were heated, centrifuged and supernatants were run on 15% polyacrylamide gel, transferred onto nitrocellose membranes and immunoblotted using SAF 84 monoclonal antibodies.

‡ The mixtures were subjected to vortex stirring before incubation at 37 °C for 1 h. After addition of 100 μl Laemmli denaturing buffer, 5 min heating at 100 °C, centrifugation at 12 000 g for 5 min, supernatant were recovered for run on SDS-PAGE. Pellets were resuspended in 100 μl of 50% v/v 8 M urea and laemmli denaturing buffer. After vigorous vortex stirring, 5 min. heating at 100 °C, 5 min centrifugation at 12 000 g the second supernatants (corresponding to re-solubilised material from pellets) were collected and run on SDS-PAGE.

§ All the tubes were incubated at 37 °C for 1 h and then 100 μl Laemmli denaturing buffer was added to the tubes of the first set. After 5 min heating at 100 °C, 2 min centrifugation, the supernatants were saved for deposition on SDS-PAGE. The tubes of the second and third sets were centrifuged, the supernatants were discarded and 100 μl of 50% v/v 8 M urea and Laemmli denaturing buffer was added per tube. After vigorous vortexing, 5 min heating at 100 °C, 2 min centrifugation at 12 000 g the second supernatants (corresponding to re-solubilised material from pellets) were collected for deposition on SDS-PAGE.

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