The Discovery of a Particle Unique to Brain and Spleen Subcellular Fractions from Scrapie-infected Mice

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SUMMARY

Fresh mouse scrapie brain and frozen mouse scrapie spleen were homogenized, sized by ultrafiltration to a fraction containing particles of 30 nm to 200 nm and separated by zonal centrifugation in sucrose gradients. In both the brain and spleen, the maximum titre of scrapie infectivity banded between densities of 1.125 to 1.200 g/ml; but in spleen a second fraction of scrapie infectivity was observed between 1.200 to 1.250 g/ml. A unique 30 to 60 nm particle was found in mouse spleen gradient fractions with high scrapie infectivity titres. This particle was not observed in similar fractions isolated from normal mouse spleen or brain and was rarely found in subcellular fractions of low titre from scrapie-infected tissues. The particle was first observed by negative staining and then confirmed in thin sections after en bloc staining with ruthenium red and potassium permanganate. The previously observed 'smearing' of scrapie infectivity over broad ranges in sucrose density gradients may now be explained since these particles were often found as aggregates or chains of particles. The significance of these particles in scrapie infectivity remains uncertain at this time, but absence of these structures in normal tissue fractions may provide a promising new morphological approach to the purification of the infective scrapie agent.

In an earlier study on the subcellular fractionation of scrapie infected mouse brain the infectious agent was observed to be closely associated with vesicular membrane components or perhaps was itself an integral part of such membranes (Siakotos et al. 1976). Clark & Millson (1976) found that scrapie infectivity was part of the plasma membrane fractions of cell cultures of scrapie-infected mouse brain, and that the vesicular microsome or the endoplasmic reticulum fraction was relatively free of the infectious agent. Clark & Haig (1970) had previously provided evidence that suggested that agent multiplication was closely involved with cell division and cell membrane proliferation, again supporting the view that the infective unit of scrapie was a part of the plasma membrane. In contrast, Prusiner et al. (1977) has reported that in the spleen the scrapie agent may be part of a large amorphous lipoprotein, separate and distinct from membranous elements, with unique sedimentation properties (Prusiner et al. 1978). In this report, the results of two fractionation experiments, one on fresh scrapie-infected mouse brain and the second on frozen scrapie-infected mouse spleen are presented.

Our general approach to subcellular and purification fractionation has been described previously (Siakotos et al. 1976). Whole brains were collected from NIH general purpose Swiss mice clinically affected with scrapie 4 to 5 months after intracerebral injection with the Compton strain (SCGM9 +10 +11) of scrapie virus. In Expt. 3, infected brains were pooled and processed immediately without freezing. For Expt. 4, spleens from infected mice were pooled, frozen in liquid nitrogen and stored frozen at -70 °C prior to fractionation. All solutions and media were the same as employed in our previous publication (Siakotos et al. 1976). In Expt. 3, 8-9 g of fresh mouse brains were pooled; for Expt. 4, 26.5 g of pooled
frozen mouse spleens were used. In both experiments the samples were homogenized, ultrafiltered, fractionated and the scrapie infectivity titres of the isolated subcellular fractions assayed as described for Expt. 2 in our earlier report (Siakotos et al. 1976). The error in the titration of the previous studies was estimated to be ±0.25 log₁₀ units (Siakotos et al. 1976), but is probably closer to ±0.50 log₁₀ units.

In Fig. 1 the infectivity data were plotted as the log titre of mouse i.e. LD₅₀/ml against fraction densities in g/ml. The scrapie infectivity data in Fig. 1 were also converted and plotted as moving three point averages to reduce the experimental variation inherent in the infectivity titration. In brain and spleen the major fraction of scrapie infectivity was localized within a broad density region of 1.25 to 1.200 g/ml. In spleen a second fraction of infectivity was observed between 1.200 and 1.250 g/ml.

In our earlier study we suggested that the re-circulating filtration system unique to the Amicon TC-3D system may be a very reliable means of sizing the infective scrapie agent (Siakotos et al. 1976). This view is supported by the data obtained in Expt. 4 with spleen, where two peaks of infectivity data were obtained with Amicon TC-3D sized material (Fig. 1). The overall absolute recoveries of the total scrapie infectious units within each complete experiment, from the initial tissue whole homogenate to the final gradient separation, were: Expt. 1, 1 %; Expt. 2, 17 %; and Expt. 3, 12 %. The differences in recoveries between Expt. 2 and 3 were probably due to incomplete washing of the material retained on the 200 nm filter. The recovery of the total scrapie infectious units applied to the zonal gradients in three brain experiments was as follows: Expt. 1, 11 %; Expt. 2, 20 % (Siakotos et al. 1976) and in Expt. 3, 75 % (this paper). In the brain experiment, the final specific infectivity i.e. log₁₀ LD₅₀/mg protein, 10⁻¹, did not exceed the initial specific infectivity of 10⁻². In marked contrast, the purification of scrapie infectivity from spleen, even from a lower
starting specific activity of $10^{5.0}$ resulted in an increase in the final preparation to $10^{7.1}$ or 150-fold purification over the starting titre.

Fractions were examined by electron microscopy across the gradient with more frequent sampling in areas of high infectivity. Electron micrographs of each fraction of scrapie infected brain or spleen were compared to a similar fraction from normal tissue. Each sample was pre-fixed for 1 h in cold 1.3% glutaraldehyde, 0.5% formaldehyde buffered with 0.05 M-collidine, pH 7.4 (Bennett & Luft, 1959). The fixed samples were then diluted 10 to 20-fold with cold distilled water, centrifuged at 150,000 g for 90 min and washed twice with water. Samples were taken for negative staining. Droplets of these samples were applied to carbon-coated grids and washed with 1% methylamine tungstate containing 0.01% bovine serum albumin (Oliver, 1973).

Selective positive staining was carried out by four methods on samples of the same pre-fixed material. One set was post-fixed by adding 100 µl saturated osmic acid and 0.5 mg ruthenium red per ml (Luft, 1971a, b), pelleted after 1 h and washed with water until free of reagents. The pellets were then stained en bloc with 1% aqueous uranium acetate (Huxley & Zubay, 1961), rinsed free of uranium acetate, dehydrated in a graded ethanol series, transferred to acetone and embedded in Spurr's standard low viscosity epoxy mixture (Spurr, 1969). A second group of samples of pre-fixed pellets were post-stained with 1% potassium permanganate for 30 min, washed with water until free of permanganate, dehydrated and embedded as above (Mollenhauer, 1959). A third sample was washed with cold water and then post-stained in 2% phosphotungstic acid, pH 2.0 (PTA), dehydrated in ethanol, washed with 0.01 N-sodium hydroxide in ethanol (Dermer, 1973) and embedded as above. A final sample of the aldehyde-osmium fixed material was stained with lanthanum by incorporating 1% La(NO$_3$)$_3$.6H$_2$O in the aldehyde and osmium fixation steps, the en bloc staining with aqueous uranyl acetate and alcohol dehydration through the 90% ethanol stage (Doggenweiler & Frenk, 1965) and embedded as above. The purpose of this was to develop a staining procedure for the scrapie specific material, since Narang (1974) had earlier suggested osmic acid-ruthenium staining to visualize scrapie specific structures. The pellets from each of the above four positive staining procedures were sectioned and the images compared with each other and to subcellular fractions isolated from normal age-matched control mice, and also to photomicrographs of negatively stained material.

Initially, an electron microscopic survey of the gradient profiles of the spleen extracts by conventional positive staining revealed only vague outlines of chains of 30 to 60 nm particles. The presence and outlines of these particles were confirmed by negative staining samples of these same preparations (Fig. 2a, c). The negative stained samples showed large fields of single particles, generally about 30 nm in diam. and occasional clusters or chains of such particles (Fig. 2a, c). Occasionally the 30 to 40 nm particles were observed in association with smaller particles, some 8 to 10 nm diam., others 16 to 20 nm. Often the 30 nm particles were observed with larger 50 to 60 nm particles (Fig. 2c). The normal spleen fractions contained only what appeared to be pieces of membrane and aggregations of such fragments (Fig. 2b).

The four sets of thin sections stained specifically for acid mycopolysaccharides and glycoproteins showed the same type of image. Both ruthenium red (Fig. 2d) and potassium permanganate showed 30 to 40 nm particles in strings and clusters, and aggregates of 50 to 60 nm. The lanthanum and acid PTA stains revealed such particles, but not as well. In all cases, normal spleen material stained lightly and contained only lightly stained membrane fragments (Fig. 2e). These unique structures were found only in fractions from scrapie infected tissue in numbers which appeared to be correlated with the relative infectivity titre of the fractions both in brain and spleen, although the particles were not as easily detected in brain. The particles were rarely found in fractions of low infectivity and not seen in any
Fig. 2. Electron micrographs of negatively stained (methylamine tungstate) preparations of fraction 24 isolated from scrapie-infected mouse spleen (a, b) and fraction 22, isolated from normal mouse spleen (c). Both fractions were matched at densities of 1.160 g/ml. (a) Scrapie-infected mouse spleen, field of mainly 30 to 40 nm particles associated with smaller structures, negatively stained; (b) normal mouse spleen, membrane fragments and aggregates, negatively stained; (c) scrapie-infected spleen, aggregates of mostly 30 to 40 nm particles associated with 10 and 16 to 20 nm particles, negatively stained. (d) Thin sections of positively stained (ruthenium red) preparations of fraction 24 of scrapie mouse spleen and (e) fraction 22, normal mouse spleen; (d) scrapie-infected mouse spleen, apparent aggregates of 30 nm particles to give clusters of 60 nm, positive stain; (e) normal mouse spleen, membrane particles, positive stain.
fractions from normal tissues. Both the morphology and the staining of these particles by ruthenium red or potassium permanganate was unique and specific. Conventionally stained thin sections showed vague chains of 30 to 60 nm particles, but the intensity of the stain was similar to the background material. The differential stain between the scrapie specific particles and background and the material in control sections was striking.

The morphology of these unique particles is complex. The 30 to 40 nm particle appears to be the basic unit. These 30 to 60 nm particle size ranges are well within the limits as defined earlier in filtration studies (Gibbs, 1967). Unpublished data from our laboratory showed that the bulk of the scrapie infectivity passed 200 nm filters but was retained by 30 nm filters. It is well known that Nucleopore filters will pass larger flexible particles because of the very thin, 10 μm polycarbonate filtering substrate. The identification of strings of particles by all methods of staining and their absence in normal tissue indicates the source of a major problem in the purification of the scrapie agent, i.e. a broad distribution of infectivity about a mean density. In the past, this scattered distribution phenomenon was considered to be the result of adsorption of the scrapie agent on other particulates (Siakotos et al. 1976), but it is now apparent that the scattered distribution of scrapie specific particles may be explained by the occurrence of chains or aggregates of particles. Unless these chains of particles were dispersed, their distribution would follow a mean density of the predominant aggregate.

These particles could be identified in thin sections only when stained specifically for acid mucopolysaccharides or acid mucoproteins (Mollenhauer, 1959; Doggenweiler & Frenk, 1965; Luft, 1971a, b; Dermer, 1973). The unique staining properties of these particles support the views of Narang (1974) that the infective agent may contain a nucleic acid core surrounded by a coat of acid polysaccharide. Narang (1974) showed that 'scrapie specific particles' in scrapie infected mouse brain could be only identified by ruthenium red or lanthanum nitrate staining of their acid mucopolysaccharide coat. The involvement of polysaccharides or glycoprotein in scrapie infection has been suggested by previous biochemical studies (Adams et al. 1969; Hunter & Millson, 1973; Suckling & Hunter, 1974).

Currently we are purifying the scrapie specific particles from scrapie infected tissues, determining the infectivity of these particles by i.c. inoculation into mouse brain and improving the resolution of the ultrastructure of the particle. Success would permit isolation and monitoring of the agent by morphological criteria, greatly facilitating subsequent studies on the scrapie agent.

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