The Membrane Location of Scrapie Infectivity

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SUMMARY

Using 5'-nucleotidase and NADPH:cytochrome c reductase as respective enzyme markers for the plasma membrane and endoplasmic reticulum, a satisfactory separation of these two membrane fractions from a cell line (SMB) derived from a scrapie mouse brain has been achieved. The coincident distribution of scrapie infectivity and 5'-nucleotidase in various fractions isolated from these cells indicates that most of the scrapie infectivity present in this cell line is associated with the plasma membrane.

The association of scrapie infectivity with membrane fractions of infected cells is well established (Hunter, Millson & Meek, 1964; Mould, Smith & Dawson, 1965). Further studies on the distribution of the agent in subcellular fractions isolated from brain and spleen have shown that a large proportion of the infectivity is associated with preparations rich in plasma membrane and endoplasmic reticulum (Millson, Hunter & Kimberlin, 1971). However, in these earlier studies resolution of the plasma membrane and endoplasmic reticulum was not sufficiently precise to ascribe scrapie infectivity to any one specific fraction.

Whilst cell cultures have not yet been infected with scrapie agent in vitro, a cell line (SMB) which originated from the brain of a mouse affected with experimental scrapie has been obtained. This line has been maintained in culture for many years and supports replication of the agent (Clarke & Haig, 1970). In recent years a number of methods have been developed for the isolation of plasma membrane and endoplasmic reticulum from different cell types (Wallach & Kamat, 1964; Bosmann, Hagopian & Eylar, 1968; Avruch & Wallach, 1971; Bingham & Burke, 1972). It therefore seemed possible that application of a cellular fractionation technique to SMB cells in vitro might yield preparations purer than had previously been obtained from either brain or spleen in vivo. This paper presents results on the distribution of scrapie infectivity in the plasma membrane and endoplasmic reticulum isolated from these cells.

The technique used was based on a method of Bingham & Burke (1972) who found that gradients of dextran T40 containing 0.5 mM-Mg$^{2+}$ gave good separation of the two types of membrane from crude microsomal fractions of chick embryo fibroblasts. In our experience the method is capable of giving good preparations of plasma membranes and endoplasmic reticulum from SMB cells when the concentration of Mg$^{2+}$ ions in the gradient is between 0.1 and 0.25 mM but the purity of each fraction is variable.

SMB cells were grown to confluency in 20 oz medical flat bottles using Medium 199 (Wellcome Reagents Ltd, Beckenham, Kent) with 10% calf serum added. The cells were harvested by treatment of the monolayers with 0.025% trypsin (Difco Laboratories, West Molesey, Surrey) and 0.02% EDTA in phosphate buffered saline, sedimented by centrifuging at 500 g for 10 min and washed once in physiological saline.

The washed cells were carefully dispersed in 1 ml of 2 M-sucrose and left at 4°C for 1 h before the addition of 7 ml of 0.01 M-tris-HCl buffer, pH 7.5. Disruption of the cells was achieved by homogenization in a Potter type homogenizer with a Teflon pestle using 30 up and down movements of the pestle. Derivation of the various fractions obtained is shown in
Short communication

Homogenized SMB cells

500g for 10 min

Pellet-crude nuclear fraction

500g for 10 min

Pellet-nuclear fraction

Post-nuclear supernatant fluids

7500g for 10 min

Pellet-crude mitochondrial fraction

50000g for 2 h

Pellet-mitochondrial fraction

Post-mitochondrial supernatant fluids

150000g for 90 min

Pellet-crude microsomal fraction

Supernatant fluid discarded

75000g for 16 h

Plasma membrane fraction and endoplasmic reticulum fraction

Fig. 1. Flow sheet to illustrate derivation of various fractions obtained from cultures of SMB cells.

Fig. 1. Following disruption the homogenate was centrifuged at 500 g for 10 min at 4 °C to sediment nuclei and unbroken cells. After resuspending and lightly homogenizing this fraction in 0.01 M-tris-HCl buffer, pH 7.5, containing 0.25 M-sucrose the preparation was further subjected to centrifugation at 500 g for 10 min, the resulting pellet constituting the nuclear fraction. Both post-nuclear supernatant fluids were combined and mitochondria sedimented by centrifuging at 7500 g for 10 min. The mitochondria were then resuspended in 0.25 M-sucrose and sedimented through 25 ml of 1.2 M-sucrose by centrifuging at 50000 g for 2 h in the SW 25.1 rotor of the Beckman Spinco Model L ultracentrifuge.

In experiment 1 both post-mitochondrial supernatant fluids were combined and a crude microsomal fraction containing endoplasmic reticulum and plasma membrane was sedimented by centrifuging at 150000 g for 90 min. In experiments 2 and 3 only the first post-mitochondrial supernatant fluid was used to obtain this fraction. The crude microsomal fraction containing endoplasmic reticulum and plasma membrane was suspended and homogenized in 1 ml of 0.01 M-tris-HCl buffer, pH 7.5, containing 0.5 mM-MgCl₂. The sample was overlayed on to a dextran gradient consisting of 0.5 ml of 40 % (w/w) dextran T40 (Pharmacia Ltd, Uppsala, Sweden) and 4 ml of 25 % (w/w) dextran T40 prepared in a 2 × ½" cellulose nitrate tube used in the Beckman Spinco SW39 rotor. The dextran solutions were prepared in 0.1 M-tris-HCl buffer, pH 7.5, containing 0.1 mM-MgCl₂.

After centrifuging at 75000 g for 16 h two membrane fractions were obtained; one at the sample −25 % dextran interface and the other at the interface between the 25 and 40 % dextran. Both fractions were collected, diluted with distilled water and centrifuged at 150000 g for 90 min to sediment the membranes.
Preparations containing (i) nuclei, (ii) mitochondria, (iii) endoplasmic reticulum and plasma membrane fraction, were resuspended and homogenized in 1.5 ml of distilled water. These suspensions were used for the estimation of protein, enzyme activities and scrapie infectivity.

Scrapie infectivity was assayed by intracerebral inoculation of 0.025 ml of tenfold dilutions into groups of 6 LAC/G mice, a method shown to give reproducible results (Millson, Hunter & Kimberlin, 1976). Scrapie was diagnosed as described previously (Hunter, Millson & Chandler, 1963), the LD$_{50}$ values being calculated according to the Kärber method (Parker, 1961) and related back to the vol. in which the fractions were suspended (1.5 ml).

The two enzymes 5'-nucleotidase and NADPH:cytochrome c reductase were employed as markers for plasma membrane and endoplasmic reticulum respectively. NADPH:cytochrome c reductase (EC. 1.6.2.3) was determined by the method of Ragnotti, Lawford & Campbell (1969) and 5'-nucleotidase (adenosine-5-monophosphatase, EC. 3.1.3.5) was determined by the method of Michell & Hawthorne (1965). The succinate 2-(p-iiodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium reductase (succinate-INT-reductase, EC. 1.3.99.1) method of Pennington (1961) was used to assay for the presence of mitochondria. Protein was estimated according to Lowry et al. (1951).

In experiment 1 when 1.1 x $10^8$ SMB cells were used a titre of 6.0 log$_{10}$ LD$_{50}$ was observed, when the cell suspension was assayed after the preliminary homogenization, equal to about 100 cells/LD$_{50}$ as previously described (Clarke & Haig, 1970) and recovery of infectivity (Table 1) from the four fractions prepared in this experiment was 67%. In experiments 2 and 3, 2 x $10^7$ and 1.6 x $10^8$ cells were used respectively. The results of the first experiment (Table 1) showed that 5'-nucleotidase, NADPH:cytochrome c reductase and scrapie activity were present in all of the major subcellular membrane fractions isolated from the SMB cells. However, the distribution of scrapie infectivity closely paralleled the activity of 5'-nucleotidase in each fraction with 47% of the scrapie infectivity and 41.7% of the 5'-nucleotidase activity associated with the plasma membrane fraction. In contrast, this fraction contained only 6.4% of the endoplasmic reticulum marker NADPH:cytochrome c reductase. The degree of mitochondrial contamination in the plasma membrane fraction, determined from the succinate-INT-reductase activity was negligible; only 3% of the enzyme activity of the crude microsomes was detected in the combined plasma membrane and endoplasmic reticulum fractions. A further 12% of the enzyme was present in the nuclear and 85% in the mitochondrial fractions.

The results of two further experiments, which gave better separation of the plasma membrane and endoplasmic reticulum, are shown in Table 1. Both fractions were better than 80% pure in respect of the 5'-nucleotidase and NADPH:cytochrome c reductase markers and in each experiment the infectivity titre and the relative specific activity of 5'-nucleotidase were about tenfold higher in the plasma membrane than in the endoplasmic reticulum (Table 1)– an observation which again suggests a correlation between scrapie infectivity and 5'-nucleotidase indicating an association between the agent and plasma membrane.

In recent years much attention has been focused on the physiological and biochemical function of the plasma membrane of the cell and consequently methods have been developed for the isolation of highly purified preparations of the membrane fraction of the cell. In many of these studies 5'-nucleotidase and NADPH:cytochrome c reductase have been shown to be specific membrane markers of the plasma membrane and endoplasmic reticulum respectively in a number of cell types. Our conclusions rest on the assumption that this is also true for the SMB cells used in this study.

No attempt was made in this study to obtain the nuclei and mitochondria in a highly purified state. Earlier work (Millson et al. 1971) showed that as nuclei, mitochondria,
Table 1. Distribution of scrapie infectivity, $5'$-nucleotidase and NADPH: cytochrome c reductase in subcellular fractions from SMB cells

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Scrapie infectivity</th>
<th>5'-nucleotidase</th>
<th>NADPH: cytochrome c reductase</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Titre $\times 10^{-5}$</td>
<td>RSA $\times 10^{-4}$</td>
<td>Units</td>
<td>%</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>5.0</td>
<td>1.00</td>
<td>14.9</td>
<td>1.49</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>5.1</td>
<td>1.26</td>
<td>18.9</td>
<td>2.17</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>5.1</td>
<td>1.26</td>
<td>18.9</td>
<td>1.75</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>5.5</td>
<td>3.16</td>
<td>47.3</td>
<td>13.2</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>4.3</td>
<td>0.20</td>
<td>6.0</td>
<td>1.27</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>5.5</td>
<td>3.16</td>
<td>94.0</td>
<td>27.0</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>5.5</td>
<td>3.16</td>
<td>20.0</td>
<td>4.21</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>6.1</td>
<td>12.6</td>
<td>80.0</td>
<td>45.0</td>
</tr>
</tbody>
</table>

* The scrapie infectivity, enzyme activities and protein content of each fraction represents the amount present in a volume of 1.5 ml. The percentage distribution of the various activities were obtained from the total of the fractions in each experiment. Relative specific activities (RSA) are given per mg of protein and 1 unit of enzyme activity = 1 nmol of substrate utilized per minute.

† NT = not tested.
myelin and to some extent lysosomes were progressively purified, the scrapie infectivity associated with them was progressively reduced thus suggesting that the activity associated with these organelles probably resulted from contamination with other membrane fragments such as plasma membrane, endoplasmic reticulum and synaptosomes. In the first experiment reported here the distribution of the enzyme markers show that there is clearly a considerable contamination of the nuclear and mitochondrial fractions with both plasma membrane and endoplasmic reticulum. However, the coincidence of the distribution of scrapie infectivity with 5'-nucleotidase suggests that the agent is more closely associated with plasma membrane than with endoplasmic reticulum. This association is even more apparent in the two further experiments when even better separation of the fractions were obtained. In view of the original derivation of the cell line, SMB, from the brain of a mouse affected with experimental scrapie it seems likely that scrapie infectivity is also associated with the plasma membrane in the brains and other tissues of affected mice.

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**REFERENCES**


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