Further characterization of scrapie replication in PC12 cells

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The rat pheochromocytoma cell line, PC12, undergoes neuron-like morphological, biochemical and electrophysiological differentiation, in the presence of low concentrations of nerve growth factor (NGF). NGF-treated PC12 cells have been shown previously to support 139A scrapie agent replication. In the present report we extended these findings and analysed the cellular conditions necessary for agent replication. Following the infection of differentiated PC12 cells, scrapie replicated to relatively high titres as determined by an incubation period assay. The removal of NGF, which causes the gradual dedifferentiation of PC12 cells, resulted in the inability of scrapie to replicate. The scrapie infectivity detected in PC12 cultures is cell-associated and not released into the medium. Cells in infected cultures did not show any change in morphology when compared to cells in mock-infected cultures. Titration studies of scrapie infectivity in PC12 cells have indicated that up to 4 LD₅₀ units per cell can be obtained although a yield of 1 LD₅₀ per cell was more common. Using an approximate m.o.i. of 1, only differentiated PC12 cells supported 139A scrapie agent replication when compared to two other differentiated, neuronal cell types, indicating that PC12 cells are more susceptible to agent replication. These studies support further the suitability of using differentiated PC12 cells as an in vitro model to study scrapie agent replication.

Introduction

Scrapie is a progressive neurodegenerative disease occurring naturally in sheep and goats. The agent can be transmitted experimentally to mice and hamsters, and these serve as useful laboratory model systems to study the disease. At least six other diseases of animals and humans are caused by similar agents: Creutzfeldt-Jakob disease, Gerstmann-Strassler syndrome, kuru, transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, and bovine spongiform encephalopathy. Disease-specific structures termed scrapie-associated fibrils and the protease-resistant protein (PrP), which is a major component of these structures, are uniquely associated with these diseases. Controversy exists concerning the nature of the agent, and three major hypotheses for the agent have been proposed: virus, prion and virino (Carp et al., 1985). The establishment of a tissue culture system which would support scrapie agent replication might enhance our understanding of the nature of the agent and would help in deciphering the neuropathogenesis associated with this and related diseases.

There have been numerous attempts to establish scrapie replication in cell culture systems. These studies used cell cultures derived from infected brains (Clarke & Haig, 1970a, b; Yanagihara et al., 1980) or infection of established cell lines with a source of agent (this has usually been diluted scrapie brain homogenate but partially purified material has been used more recently) (Gibson et al., 1972; Clarke & Millson, 1976; Clarke, 1979; Yanagihara et al., 1980; Race et al., 1987; Butler et al., 1988). In all cases, the cells were mitotically active throughout the experiments. These systems yielded unimpressive infectivity titres, generally 1 LD₅₀ per 100 to 1000 cells. The poor levels of replication may be a consequence of several factors including the cell types employed or the rate of cell division. These latter two factors have been addressed in the PC12 cell culture system.

Previously we have reported a neuron-like cell which is capable of supporting scrapie agent replication (Rubenstein et al., 1984). This cell line, termed PC12, is a cloned tumour cell line derived from a rat pheochromocytoma. In the continued presence of nerve growth factor (NGF) these cells cease dividing and acquire the morphological, biochemical and electrophysiological properties of differentiated neurons (Greene & Tischler, 1976; Greene & Rein, 1977a, b, c; Reiger et al., 1980; Rudy et al., 1982). In this report we extend our studies on scrapie-infected PC12 cells and demonstrate not only that the scrapie agent is capable of replicating in these cells to
higher titres than any other reported cell line but also that the neuron-like state of the cell plays a determining role in controlling scrapie replication.

**Methods**

**Cells and medium.** PC12 cells were maintained and passaged as described previously (Rubenstein et al., 1984). Briefly, cells were grown in RPMI 1640 medium containing 10% heat-inactivated horse serum (Gibco) and 5% foetal calf serum (Gibco). Cells were maintained at 37 °C in a 95% air, 5% CO₂ atmosphere and were re-fed and subcultured weekly. Differentiation was initiated by first plating cells in 35 mm² plastic tissue culture dishes (10⁶ to 5 × 10⁸ cells/dish) previously coated with air-dried rat tail collagen (Bornstein, 1958). These cells were maintained in 2 ml of the above medium supplemented with 100 ng/ml 2.5S NGF (Mobley et al., 1976). Cells were maintained in the presence of NGF for 2 to 3 weeks prior to scrapie agent exposure.

The cloned human neuroblastoma cell line, SH-SY5Y, was provided by Dr June Biedler, Sloan Kettering Cancer Center, New York, U.S.A. These cells were cultured in the same medium used for PC12 cells. Differentiation of SH-SY5Y cells was induced by exposure to 10⁻⁵ M-cis-retinoic acid in 0.1% ethanol (Cole et al., 1985). Medium was changed twice a week and cells were allowed to differentiate for 3 weeks prior to infection. The mouse neuroblastoma cell line, Neuro 2A, was obtained from the ATCC. Cells were maintained in Eagle's MEM supplemented with 10% foetal calf serum. These cells were induced to differentiate by the addition of 1 mm-dibutyryl cAMP (Daniels & Hамprechт, 1974) and cultures were maintained in this state for 3 weeks prior to infection.

**Preparation of inoculum and infection of cells.** The cloned 139A scrapie agent (kindly provided by Dr R. H. Kimberlin, AFRC Neuropathogenesis Unit, Edinburgh, U.K.) was passaged by intracerebral (i.c.) injection into C57BL/6j mice. Animals showing signs of clinical disease (Carp et al., 1984) were sacrificed and a 10% brain homogenate was prepared in phosphate-buffered saline and stored at −70 °C. The titre of a typical 139A scrapie brain was approximately 10⁸⁵ LD₅₀ per gram. The inoculum for the in vitro studies was prepared by diluting the homogenate further with medium such that an m.o.i. (based on the ratio of LD₅₀ units per cell) of 1 was obtained (usually this involved further diluting the 10⁸⁵ brain homogenate 100-fold, thereby infecting the cultures with approximately 10⁷ LD₅₀culture). Brain homogenate from normal animals was prepared similarly and diluted for control studies.

Cultures were infected by removing the old medium and adding 1 ml of the diluted brain homogenate. Following incubation at 37 °C for 5 h the inoculum was removed, cultures were washed twice, and fresh medium was added. Unless otherwise indicated, cultures were re-fed twice a week with growth medium containing NGF.

**Preparation of samples for bioassay.** In most experiments cultures were harvested as described previously (Rubenstein et al., 1984). In brief, at each time point cultures (cells and medium together) were frozen and thawed three times, cell fragments were scraped from the dish, and then both disrupted cells and medium were transferred to a sterile tube, and bio assayed. The freezing and thawing procedure resulted in the complete disruption of all cells. In experiments in which the infectivity in cells and medium was assayed separately, the medium from separate cultures was removed at each refeeding, pooled with that from previous refeedings of that culture, and stored at −70 °C. At the termination of the experiment the medium was separated from cellular debris by centrifugation at 1000 g for 15 min, concentrated to one-tenth of its original volume by lyophilization, frozen and thawed three times, and bioassayed. The cells from these studies were washed twice with fresh medium, scraped from the surface of the tissue culture dish, transferred to a sterile tube, frozen and thawed three times, and bioassayed.

**Determination of scrapie infectivity.** The level of scrapie infectivity was measured either by incubation period assays (Hunter et al., 1963; Chandler, 1963) or by endpoint titration assays (Mould et al., 1967). Bioassays on each sample were performed by injecting 30 μl of each dilution i.c. into six C57BL/6j or Compton White mice. Scrapie-induced clinical disease was measured by observing mice on a grid system as described previously (Carp et al., 1984). Scrapie incubation periods were determined from the date of i.c. injection to the time mice exhibited signs of clinical disease for 3 consecutive weeks. Values for the average incubation period were calculated only if greater than 50% of the animals exhibited clinical signs of scrapie. Decreases in incubation period are indicative of an increase in the level of scrapie infectivity. Mice exhibiting no signs of disease by 250 days post-injection were scored as negative.

**Results**

Since the 139A scrapie agent is able to replicate in NGF-treated PC12 cells (Rubenstein et al., 1984), we now examined whether the differentiated state of the cell was a prerequisite for replication of the agent. NGF-treated PC12 cells were infected at a multiplicity of 1 with diluted 139A scrapie brain homogenate. Cultures were harvested at different times post-infection (p.i.) and infectivity was determined by the incubation period assay described in Methods. The incubation periods for the undiluted samples became shorter as the time after infection increased, and this continued until the end of the experiment (Table 1). For the majority of these time points most of the animals inoculated with the samples developed clinical disease indicating the presence of high levels of infectivity and therefore scrapie agent replication. When the samples were diluted 10-fold and then bioassayed, only the samples at 49, 63 and 70 days p.i. showed a high percentage of animals with clinical disease and therefore incubation periods were determined only for these time points (Table 1). Incubation periods were determined for the clinically positive animals only. Since not all the animals from the 49 and 63 day time points showed clinical disease by 250 days post-injection, the actual incubation periods are longer. Despite this evidence for high levels of replicating agent, the PC12 cells did not exhibit any cytopathic effects throughout the course of the experiment. Bioassays on 10-fold diluted samples from 7 to 35 days p.i. were attempted also. The small number of animals that showed signs of clinical disease indicated that the infectivity levels were very low and therefore incubation periods could not be determined. These results support our previous findings that the agent replicates in NGF-treated PC12 cells.
To find out the effects of differentiation on the ability of scrapie agent to replicate, we infected NGF-treated PC12 cells and removed the NGF 1 week after infection (day 7, Table 1). After NGF was removed, the cells gradually lost their neuronal properties over a period of 2 to 3 weeks. By 3 weeks after the removal of NGF, scrapie agent replication was not detected, as seen by the low percentage of clinical animals and the long incubation periods (Table 1). These results indicate that PC12 cells must be in the differentiated state to support scrapie replication.

We measured infectivity by the incubation period assay in cell fractions and medium separately to determine whether the agent remains cell-associated or is released into the medium. Differentiated PC12 cells were infected with the 139A scrapie brain homogenate. At various times p.i. cultures were harvested, the cells and medium were separated as described in Methods, and infectivity was determined in each fraction by incubation period assays. When the cell fractions were examined (Table 2) there was no decrease in the incubation periods, i.e. increase in the amount of scrapie infectivity, from 0 to 20 days p.i. The levels of infectivity increased significantly by 27 and 34 days p.i. The cells at these time points contained more infectivity, at least 1000-fold, than the amount found in medium. Bioassays on the concentrated medium showed barely detectable levels of scrapie infectivity from day 13 p.i. until the end of the experiment (Table 2). The infectivity detected at 0 and 6 days was probably due to residual inoculum from the original brain homogenate. For most of the samples of medium (days 13 to 34) only a fraction of the animals injected showed clinical signs by 250 days. Therefore, the incubation periods for these samples are underestimated. These results clearly indicate that the scrapie agent is not secreted following replication but remains cell-associated.

To obtain a more accurate measure of the amount of infectivity associated with the NGF-treated PC12 cells during the course of scrapie agent replication, we performed endpoint titration assays on cultures at various times p.i. (Table 3). The infectivity associated...
with these cultures decreased slightly until 21 days p.i. Infectivity then increased throughout the remainder of the experiment. When infectivity was determined on a per cell basis, an average of approximately 4 LD$_{50}$ was associated with each cell by 56 days p.i. Although we have found that 4 LD$_{50}$ per cell was obtainable, infectivity levels were usually closer to 1 LD$_{50}$ per cell in other experiments. It is not known whether the number of LD$_{50}$ units per cell is a function of the fact that many cells have a low number of LD$_{50}$ units or whether there is a high level of infectivity in a small number of cells with the vast majority of cells devoid of infectious agent.

The amount of time infected cells remain in culture may be an important factor in generating high yields of scrapie infectivity. As shown in Table 3, there was an approximate 2000-fold increase in scrapie titres from infected samples taken between 21 and 56 days p.i.

The question of whether high levels of scrapie replication is a general finding in neuron-related cell cultures has also been examined (Table 4). Cultures of PC12 cells, Neuro 2a and SH-SY5Y were induced to differentiate (see Methods). All three differentiated cell types were infected with the diluted 139A scrapie brain homogenate at an approximate m.o.i. of 1. Representative cultures at different times were harvested and bioassayed (Table 4) by incubation period assays. Since not all the animals developed the disease, the percentage of animals exhibiting clinical signs was determined. By day 29 p.i. 50 to 80% of the animals injected with infected samples from NGF-treated PC12 cells showed clinical signs of scrapie. From day 35 p.i. until the end of the experiment all of the animals injected with infected PC12 cells demonstrated clinical disease indicating that the levels of infectivity had increased compared to earlier times of harvest. In contrast, animals injected with Neuro 2a or SH-SY5Y cells which were exposed to the scrapie brain homogenate showed decreasing percentages of animals with clinical disease as the time after injection increased, indicating reduced amounts of scrapie infectivity. The infectivity present at the early time points was probably the result of residual scrapie brain inoculum which remained after infection. It is interesting to note the rapidity with which infectivity decreased in the Neuro 2a and SH-SY5Y cells despite their differentiated state.

### Discussion

NGF-treated PC12 cells are susceptible to scrapie agent infection and support its replication (Rubenstein et al., 1984). Our current studies help define the cellular conditions required for scrapie replication. A number of previous studies have attempted to establish a scrapie-infected tissue culture cell line using brain homogenate as the inoculum. Some of these infected cultures showed a decrease in infectivity upon continued passage until infectivity became undetectable (Yanagihara et al., 1980; Markovits et al., 1981, 1982). Other reports demonstrated detectable levels of infectivity in all cell passages (up to 150 passages for one cell line) from infected cultures (Clarke & Haig, 1970a, b; Clarke & Millson, 1976; Clarke, 1979; Cherednichenko et al., 1985; Race et al., 1987; Butler et al., 1988). Furthermore, some of these studies have reported success in establishing cloned cell lines of scrapie-infected cells (Race et al., 1988; Butler et al., 1988) whereas others have observed changes in the biological properties of scrapie-infected cells (Cherednichenko et al., 1985; Markovits et al., 1981, 1982).

PC12 cells supported scrapie replication only when in the neuronal state and the infectivity was restricted to the cellular fractions. Morphologically, infected cells could not be distinguished from control cells. The cell association of infectivity and the absence of morphological changes are similar to results reported previously (Clarke & Haig, 1970a, b; Race et al., 1987). Our studies indicate also that the continued presence of NGF is required for replication to be maintained. Removal of NGF from infected cells caused dedifferentiation, with the resumption of cell division and resulted in the inability of the scrapie agent to replicate. It is not known whether the infectivity in differentiated PC12 cells is a function of the specific cell type, or results from the fact that the PC12 cells are non-dividing, or is due to the intracellular environment established by NGF, or any combination of the above. The answer to this may be found by infecting other cell types which respond similarly to NGF and comparing the levels of infectivity.
to that obtained in PC12 cultures. As only the differentiated PC12 cells can support an increase in infectivity, it can be speculated that the neurites may be a site for the accumulation and/or replication of scrapie agent.

Previously scrapie agent has been reported to replicate in a number of dividing, non-differentiated cell lines; therefore, the important issue is to determine what is different about NGF-treated PC12 cells. It is known that exposure of PC12 cells to NGF causes many physiological changes in these cells (Greene & Tischler, 1976; Greene & Rein, 1977a, b, c; Dichter et al., 1977). It is uncertain whether the neuron-like state of the cell and/or other NGF-induced cellular changes are responsible for creating the proper intracellular conditions to allow scrapie agent replication. In addition, it has been shown previously that the addition of NGF to PC12 cells causes stimulation in the transcription of many genes including, but not limited to, those encoding actin and ornithine decarboxylase, and the proto-oncogenes c-fos and c-myc (Greenberg et al., 1985; Curran & Morgan, 1985; Kruijer et al., 1985; Mildbrandt, 1986; Kujubu et al., 1987). Mобley et al. (1988) have shown that NGF causes an increase in PrP mRNA in cholinergic neurons of developing hamster brains. Furthermore, it has been reported recently (Wion et al., 1988) that NGF increased the expression of the PrP gene in PC12 cells. Whether or not this is a contributing factor for scrapie replication in these cells is not known. Western blot analysis of scrapie-infected mouse neuroblastoma cells has demonstrated the presence of scrapie-specific PrP (Butler et al., 1988). We are currently using infected PC12 cells to relate levels of infectivity to the time of appearance and accumulation of PrP.

We have demonstrated that the phenomenon of cell differentiation is not, by itself, adequate to account for the ability of scrapie agent to replicate. Under our conditions for infection, drug-induced differentiation of two cell types of neuroblast origin did not permit scrapie replication. We have found that the 139A mouse-adapted scrapie agent failed to replicate in a differentiated human neuroblastoma cell line, SH-SY5Y. In addition, the 139A scrapie agent did not replicate in a differentiated mouse neuroblastoma cell line, Neuro 2a, when infected with a multiplicity of 1. This is in agreement with previous studies (Butler et al., 1988) showing that the scrapie agent replicated in undifferentiated mouse neuroblastoma cells when infected with a multiplicity of 100 but failed to replicate following infection at a multiplicity of 1. Since an m.o.i. of 1 was used for the PC12 studies reported here, it suggests that efficiency of infection is much greater for PC12 cells than the other cell lines used. It should be pointed out that the extent of differentiation observed for Neuro 2a and SH-SY5Y was not as extensive as that obtained for PC12 cells. For both Neuro 2a and SH-SY5Y, neurite outgrowth was not as robust and, although cell division was greatly reduced, it was not completely halted. Whether these factors played a role in the ability of agent to replicate in these cell types is not known. Also it is not known whether the levels of PrP mRNA are increased following drug-induced differentiation in Neuro 2a and SH-SY5Y. A unique feature of the NGF-treated PC12 cells is the ability to maintain the cells in culture over long periods without the need for subculturing. This too, may be a contributing factor to the yields of scrapie infectivity obtained.

The maximum infectivity measured in the NGF-treated PC12 cells was found to be higher than that for any other cell line previously reported. We determined that between 1 and 4 $L_\text{D}_{50}$ was produced per cell which is much greater than reported previously for other scrapie-infected cell lines (Clarke & Haig, 1970a, b; Clarke & Millson, 1976; Race et al., 1987). In those studies yields were at best 1 $L_\text{D}_{50}$ per 100 cells (Clarke & Haig, 1970a, b). Even in those instances in which cloning of infected cells was done (Race et al., 1988; Butler et al., 1988), yields did not approach those obtained in the present study. Although we attempted to maintain similar conditions for each experiment (cell number, inoculum, infection protocol and bioassay) the amount of infectivity present in the cultures varied between experiments. The infectivity in NGF-treated PC12 cells may, in fact, be an underestimate of the infectious agent actually produced since the PC12 cells are of rat origin and we assayed for infectivity in mice. The concept of species barrier in relation to scrapie strain–host strain combinations has been well established in vivo (Kimberlin & Walker, 1978; Kimberlin et al., 1987). Whether a species barrier exists in vitro is not known but the issue will be addressed by infecting PC12 cells with a rat-adapted scrapie agent and bioassaying cell homogenates in both rats and mice.

A great deal of work has been done concerning the spread of scrapie agent in vivo (Kimberlin & Walker, 1979, 1980, 1982; Kimberlin et al., 1983a, b; Fraser, 1982; Fraser & Dickinson, 1985). Many of these studies dealt with questions concerning the mechanism of neuroinvasion and the factors controlling it for different scrapie agents and for a variety of injection routes. Previous studies on the haematogenous spread of scrapie have been performed and its role in the neuropathogenesis of the disease has been discussed (Manuelidis et al., 1978; Millson et al., 1979; Kuroda et al., 1983; Diringer, 1984). There is ample evidence suggesting that scrapie-induced pathogenesis is due to neural spread of infectivity (Buyukmihci et al., 1980, 1983; Fraser, 1982; Kimberlin & Walker 1980, 1986). Our in vitro studies on infection of PC12 cells appear to coincide with the in vivo
studies in that infectivity remains cell-associated and is not released into the medium. Studies using this in vitro system may help determine the mechanism of cell-to-cell transmission of scrapie. PC12 cells may prove to be useful in examining the role of neuronal transport in the spread of scrapie and the influence of scrapie replication on axonal transport of cellular macromolecules. This may aid in our understanding of the mechanism of scrapie-induced neuropathogenesis in vivo.

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