In vitro Replication of Scrapie Agent in a Neuronal Model: Infection of PC12 Cells

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SUMMARY

A rat phaeochromocytoma cell line, termed PC12, was used to study scrapie replication. These cells, in response to the addition of nerve growth factor (NGF), exhibit a number of neuronal properties including morphological differentiation, electrophysiological responsiveness, and neurotransmitter synthesis. Cultures were exposed to scrapie brain homogenate (strain 139A), harvested every week for up to 6 weeks, and assayed for scrapie infectivity. Scrapie replication in vitro was monitored by injecting scrapie agent-exposed NGF-treated PC12 cells into mice and measuring time intervals from injection to onset of clinical symptoms. Mouse incubation periods vary inversely with the amount of scrapie infectivity present. Cells harvested at 7 and 14 days after exposure to scrapie agent showed a decrease in the level of infectivity followed by an increase at subsequent time points. The increase in scrapie infectivity from early to late time intervals after agent exposure clearly indicated replication in vitro. A fusion agent was not necessary to establish infection, and the addition of mouse peritoneal macrophages caused a reduction in the yield of infectivity per culture. Examination of cells by phase-contrast microscopy failed to reveal any cytopathology.

INTRODUCTION

Scrapie, a central nervous system (CNS) disease affecting sheep and goats, is a prototype of the unconventional slow virus infections, a group that includes kuru and Creutzfeldt–Jacob disease in man and transmissible mink encephalopathy (Marsh, 1974; Gajdusek, 1977). The pathogenesis of scrapie has been studied in a variety of experimental models of the disease in mice (for reviews, see Outram, 1976; Kimberlin, 1976, 1979; Dickinson & Fraser, 1977; Dickinson & Outram, 1979).

There have been many attempts to culture the scrapie agent in vitro. Some of these studies involved examination of the growth characteristics of a cell line, referred to as SMB, derived from a scrapie agent-infected mouse brain (Clarke & Haig, 1970a, b). Cultures continued to yield low levels of infectious agent for at least 150 subcultivations (Clarke, 1979). The yield of agent appeared to be correlated positively with cell replication and an average of only one infectious unit was produced per cell. The cells involved in replication of scrapie were never fully characterized, but clearly neurons were not involved since they would not survive even the initial subcultivations.

It was also found that cell cultures could be established more readily from scrapie-infected mouse brains than from normal mouse brains (Field & Windsor, 1965; Gustafson & Kanitz, 1965; Haig & Pattison, 1967). In other studies, scrapie agent replication was reported following infection of glial and neuroblastoma cell lines in vitro (Markovits et al., 1981, 1982). After 12 to 16 passages these authors also noted altered morphology and growth characteristics of the infected cells. This work has not been repeated thus far.
Attempts to demonstrate replication of infectious agent and/or cytopathic changes in non-neuronal cells exposed to agent \textit{in vitro} have yielded only limited success. In a series of studies, mouse L cell fibroblasts were exposed to agent \textit{in vitro} either with or without lysolecithin treatment of the scrapie brain homogenate (Clarke & Millson, 1976; Clarke, 1979). Although infectious agent was produced by these cells, the yield was low and with continuous subcultivation there was often a decrease in titres. These cells did not show cytopathic changes and, in fact, exposure of a wide array of tissue culture cell types has never shown a consistent, scrapie-related cytopathology (Haig & Clarke, 1965; Gibson \textit{et al}., 1972).

The only major target organ for scrapie agent is the nervous system. In addition, the nerve cell may play a role in transport of agent from peripheral sites of infection such as the peritoneal cavity (Kimberlin & Walker, 1980, 1982) and the eye (Fraser, 1982; Buyukmihci \textit{et al}., 1983). It would, therefore, be appropriate to develop an \textit{in vitro} neuronal model to study this agent. We have attempted to develop such a model by using the PC12 cell line which has previously been utilized as an \textit{in vitro} model of the nervous system to study herpes simplex virus infection of neurons (Rubenstein & Price, 1983\textit{a}, \textit{b}, \textit{c}). The PC12 cells were cloned from a rat phaeochromocytoma (Greene & Tischler, 1976) and in the presence of low concentrations of nerve growth factor (NGF) cease cell division and undergo morphological, physiological and biochemical differentiation which consists of extension of neurites, development of an excitable membrane and neurotransmitter synthesis (Greene & Tischler, 1976; Greene & Rein, 1977\textit{a}, \textit{b}, \textit{c};; Reiger \textit{et al}., 1980; Rudy \textit{et al}., 1982). In this report we have successfully used the PC12 cells as an \textit{in vitro} culture system for scrapie replication.

\section*{METHODS}

\textit{PC12 cells.} PC12 cells were grown as previously described (Greene & Tischler, 1976; Rubenstein & Price, 1983\textit{c}). In brief, cells were grown in 75 cm\textsuperscript{2} plastic tissue culture flasks (Falcon) in RPMI 1640 medium (Gibco) supplemented with 10\% heat-inactivated horse serum (HS) (Biofluids, Rockville, Md., U.S.A.), 5\% foetal calf serum (FCS) (Flow Laboratories), 50 units/ml penicillin (Gibco) and 25 mg/ml streptomycin (Gibco). Cells were maintained at 37 °C in a 95\% air, 5\% CO\textsubscript{2} atmosphere and were re-fed and subcultured weekly.

Morphological differentiation was induced by first plating cells in 35 mm plastic tissue culture dishes (Falcon) coated with air-dried rat-tail collagen (Bornstein, 1958). These cells were then treated with \textit{maintenance medium} consisting of RPMI 1640, 2\% HS, and 100 ng/ml of 2.5S NGF (Mobley \textit{et al}., 1976). Cells were maintained with NGF for 3 weeks prior to scrapie agent exposure.

\textit{Peritoneal macrophages.} C57BL/6J mice were primed by intraperitoneal (i.p.) injection of 2.0 to 3.0 ml of thioglycolate. Macrophages were harvested as previously described (Carp & Callahan, 1981) and seeded 1 week prior to exposure to agent. Cells (3 × 10\textsuperscript{4} to 5 × 10\textsuperscript{4}) were plated on collagen-coated 35 mm Petri dishes in \textit{maintenance medium} in the presence or absence of NGF-treated PC12 cells.

\textit{Scrapie agent.} The original aliquot of 139A agent was kindly provided by Dr R. H. Kimberlin (ARC & MRC Neuropathogenesis Unit, Edinburgh, U.K.). The agent was passaged in C57BL/6J mice by intracerebral (i.c.) injection of 0.03 ml of a 1\% suspension. Brains were removed from infected mice at least 3 weeks after the initial scrapie symptoms. A 10\% homogenate was made in phosphate-buffered saline (PBS) containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, divided into aliquots and stored at −70 °C.

\textit{Exposure of cells to scrapie agent.} An aliquot of the 10\% 139A scrapie brain homogenate was thawed and centrifuged at 800 g for 10 min. The supernatant was used as the source of agent. The agent was diluted to 10\textsuperscript{-3} with maintenance medium and 0.5 ml was added to each culture. Cultures were incubated at 37 °C with occasional shaking. After 6 h, cultures were washed twice with PBS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and maintenance medium was added.

\textit{Cell fusion.} Cell fusion was carried out using polyethylene glycol (PEG) 1000 (Fisher Scientific Co., Fairlawn, N. J., U.S.A.) diluted in serum-free medium (or serum-free medium in the absence of PEG in the case of mock fusion) as follows: cells were washed twice with PBS then exposed to PEG (50\%, v/v) containing 0.5\% DMSO for 1 min; an equal volume of serum-containing medium (10\% HS + 5\% FCS) was slowly added and cells were washed once with serum-containing medium before addition of maintenance medium. Cultures were re-fed weekly.

\textit{Harvesting cultures.} Representative cultures were harvested by placing the Petri dish containing both cells and fresh medium at −70 °C. At the termination of each experiment all cultures (cells and medium) were frozen and thawed three times, cells scraped from the plastic surface with rubber policemen and the entire contents transferred to sterile tubes. For each sample five or six mice (Compton White or C57BL/6J) were injected i.c. with 0.03 ml and scored for clinical signs of scrapie.
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Incubation period determinations. Measurement of incubation period as a means for determining scrapie infectivity has been previously reported (Hunter et al., 1963; Dickinson et al., 1966; Carp & Callahan, 1981). With the scrapie agent-mouse strains used in the present study the earliest clinical changes noted were a decrease in both motor activity and competency. This was measured on a grid as previously described (Carp et al., 1984). Monitoring was begun 90 days post-injection and the scrapie incubation period was designated as the date when the mouse had shown clinical signs of disease for the third consecutive week. Mice exhibiting no signs of clinical disease by 250 days were scored as negative.

Statistical analysis. Since some samples exhibited large variations in their incubation periods and several groups of mice showed less than 100% mortality, we chose to represent central tendency by the median rather than the mean. Quartile deviation, calculated between the first and the third quartiles, expresses the variation among the incubation periods for each sample. To analyse differences between medians, the sign or 'median' test was used (McNemar, 1955).

RESULTS

PC12 cells grown in RPMI 1640 medium containing 10% HS and 5% FCS have a rounded shape and grow mainly in small clumps, as examined by phase-contrast microscopy (Fig. 1a). Cultures maintained in maintenance medium on collagen-coated plastic Petri dishes for 3 weeks underwent morphological differentiation exhibiting an extension of a plexus of neurites, and enlarged cell bodies containing distinct nuclei and prominent nucleoli (Fig. 1b).

Scrapie infection of PC12 cells alone was carried out on cultures (2 x 10^5 to 5 x 10^5 cells) that had been treated with NGF for 3 weeks. PC12 cells were exposed to scrapie agent as described in Methods and examined by phase-contrast microscopy. Both immediate and subsequent weekly examinations showed that these cultures were indistinguishable from both untreated cells and cells exposed to a similarly diluted supernatant from normal brain homogenate. Parallel studies were carried out on mouse peritoneal macrophages plated alone and in combination with PC12 cells. Weekly examinations of these cultures by phase-contrast microscopy again revealed no cytopathological effects as compared to controls.
Fig. 2. Median incubation periods + quartile deviation of mice injected i.c. with samples from scrapie agent-infected NGF-treated PC12 cells (a), mouse peritoneal macrophages (b), or a combination of the two cell types (c). Dotted lines represent the median incubation periods of cell samples diluted 10-fold prior to i.c. injection. Ratios indicate mortality rates (number positive for scrapie/total number injected).

Fig. 3. Changes in median incubation periods relative to the 6 h sample of cultures harvested at various times after scrapie infection in vitro. The ordinate shows the difference between median incubation periods, i.e. the median incubation period of each sample minus the median incubation period of the 6 h sample (zero line). (Median incubation period ± quartile deviation for 6 h samples: non-fused, 170 ± 19; fused, 175 ± 17.) Ratios below or above bars indicate mortality rates (number positive for scrapie/total number injected).

The scrapie infectivity studies detailed in this paper are representative of six experiments which have been carried out all yielding similar results. As can be seen in Fig. 2(a), the median incubation period for the undiluted sample of PC12 cells at the 6 h time point was 174 days. This value represented residual scrapie agent remaining in the cultures after removal of unadsorbed infectivity. At 15 days post-infection, the median incubation period increased to 188 days, indicating approximately a 10-fold decrease in the amount of infectivity present. Scrapie infectivity in the PC12 cells began to increase by 22 days post-infection as reflected by a decrease in the incubation times to 172 days. Scrapie replication continued until the end of the experiment (43 days) as evident from the relatively low incubation period of 158 days. This value was statistically different ($P < 0.025$) from the incubation period for the day 15 culture. In most of the undiluted samples assayed, 100% of the animals exhibited clinical disease (Fig. 2a). This indicates that the scrapie titre endpoint may not have been reached. When the samples were diluted 10-fold before i.c. injection into mice, the median incubation period during the first 29 days varied from 196 days for the 15 day time point to greater than 250 days for the 22 day sample. At this dilution, scrapie infectivity was significantly ($P < 0.001$) higher in the 35 and 43 day samples, which had median incubation periods of 202 and 168 days respectively, when compared to the 22 and 29 day samples (Fig. 2a). In addition, a 100% mortality rate for these latter two time points again suggests that the titration endpoint has not been reached.
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Fig. 2(b) shows the median incubation periods for mice injected i.c. with undiluted samples from scrapie-inoculated mouse peritoneal macrophages harvested at the indicated times. The 6 h time point again represents residual scrapie agent. All six animals injected were positive for scrapie with a median incubation period of 180 ± 21 days. There was virtually no scrapie infectivity present at any other time point with only one mouse positive for scrapie in the 22 day sample and none at the other time intervals. In parallel studies, 139A agent (10⁻³ dilution) was added to collagen-coated plastic Petri dishes in the absence of cells. At each time point when cells were harvested, a 0.5 ml aliquot of agent was removed and frozen in an identical manner as the cultures. The median incubation periods were assayed for undiluted samples at the 6 h, 22 and 43 day time points and found to be 150, 158 and 161 days, respectively. These results indicate a slight increase in incubation periods for the samples harvested at the later time points. Furthermore, the inactivation of scrapie agent in the presence of macrophages was greater than the inactivation of agent incubated in the absence of cells.

The infectivity results for cultures containing both NGF-treated PC12 cells and mouse peritoneal macrophages are shown in Fig. 2(c). While agent was present at the 6 h time point, relatively little or no agent was present in undiluted samples from 15 to 29 days after infection in vitro as evidenced by the long incubation periods. Shorter incubation periods at days 35 and 43 clearly indicate agent replication. Tenfold dilutions of these later two culture samples indicated that although an endpoint had been reached for the 35 day sample, the 43 day time point continued to show the presence of infectivity and 80% mortality. Four of five mice injected with a 10-fold dilution of the 43 day sample from the combined cultures (PC12 cells plus macrophages) were positive with a significantly (P < 0.025) longer median incubation period than the value obtained for a similarly diluted sample of infected PC12 cells alone at 43 days (Fig. 2a, c).

In an attempt to study the PC12 cell–macrophage interaction more closely, cell fusion studies between mixed cultures of PC12 cells and macrophages were performed as described in Methods. These cultures were fused following a 6 h exposure to the 139A scrapie agent. Fusion, monitored by phase-contrast microscopy and measured by an increase in the number of multinucleated PC12 cells, was found to occur in 20 to 25% of the cell population in each culture. The PEG did not appear to be toxic to the cultures when tested by trypan blue exclusion. Both the fused and non-fused cultures followed the same general pattern, i.e. a statistically significant (non-fused, P < 0.001; fused, P < 0.001) decrease in the incubation periods (increase in agent titre) at 28 and 35 days post-infection when compared to the 7 and 14 day time points (Fig. 3). The differences observed between the median incubation periods for the fused and non-fused samples within each time point were not statistically significant. Scrapie agent replication was evident from the data in Fig. 3 when a comparison was made between the median incubation periods of the 7 and 35 day samples. A difference of 102 days was noted for the non-fused cells and 32 days for the fused cells. Comparison of the incubation periods with standard dose–response curves for this agent–strain combination indicates an approximate increase in titre of 10⁴- to 10⁵-fold and 10²-fold, respectively.

DISCUSSION

The exposure in vitro of NGF-treated PC12 cells to mouse scrapie agent followed the classical virus replication pattern. Relatively large quantities of agent (short incubation periods) were present at 6 h post-infection. This represents residual agent that was not removed when the cells were washed. The amount of agent gradually decreased (longer incubation periods) during the first 2 or 3 weeks of incubation. Since continued incubation led to replication of agent, the reduction in infectivity within the first 3 weeks would appear to be comparable to the eclipse phase seen with conventional viruses. This profile is more pronounced in cultures containing both PC12 cells and macrophages as compared to PC12 cells alone. The controversy over the existence of a characteristic viral eclipse phase following scrapie inoculation into animals has been argued by others (Field et al., 1971; Hunter et al., 1972). The data obtained in animals can be explained by a number of events that can occur in the animal in addition to a standard eclipse phase. Early titres, suggesting the absence of an eclipse phase, might represent the persistence of
non-replicating scrapie agent that had accumulated from the large initial inoculum whereas the disappearance of the scrapie agent from a tissue might reflect a migration or sequestration of agent rather than an eclipse phase. The *in vitro* PC12 system, with only a single cell type, eliminates these problems and the results suggest the existence of a true eclipse phase following scrapie infection.

In cultures of PC12 cells alone significant scrapie replication was first detected between 3 and 4 weeks following the addition of agent. When scrapie brain homogenate was added to cultures of mouse peritoneal macrophages, scrapie agent was virtually undetectable from 15 days post-exposure until the end of the experiment (6 weeks). Inactivation of scrapie by macrophages was also evident at the 6 h time point where incubation periods were slightly longer in macrophage-containing cultures as compared to PC12 cells alone (Fig. 2). This is in agreement with previous studies by Carp & Callahan (1981, 1982) who showed that scrapie agent was associated with macrophages following incubation *in vitro* for 2 h at 37 °C. They then showed that with longer incubation *in vitro*, scrapie infectivity decreased as measured by an increase in incubation period (Carp & Callahan, 1982).

Cultures containing both PC12 cells and mouse peritoneal macrophages yielded results consistent with those obtained with PC12 cells and macrophages maintained separately. While PC12 cells presumably supported scrapie replication under these culture conditions, increased infectivity was not detectable until 35 days after cell inoculation. Furthermore, the final yield of infectivity was lower in cultures of PC12 cells plus macrophages compared to cultures with PC12 cells alone. The lag in demonstrable replication and this reduced yield are probably related to macrophage inactivation of agent. Thus, it is likely that in these cultures scrapie agent is replicating in the PC12 cells, and at the same time being inactivated by macrophages.

NGF-treated PC12 cells are non-dividing, indicating that replication of the scrapie agent does not require cell division. Since phagocytosis and membrane fusion may play a role in scrapie infectivity, experiments using the fusion agent PEG were performed in mixed cultures of PC12 cells and macrophages. Our results demonstrated that PEG-induced membrane fusion increased the uptake of agent but it was not an obligatory step for scrapie infection and subsequent replication.

Our results suggest that scrapie replication occurs in neuronal elements and that no other cell type is necessary (it is of interest to note that the PC12 cells are similar to peripheral nerve cells). Furthermore, it suggests that certain cell types, such as peritoneal macrophages can act to modulate the level of infectious agent. Although the PC12 cells appear to be a suitable *in vitro* model to study scrapie replication, a direct comparison between this system and the animal model may be premature. Dickinson & Outram (1979) have stated that sources of variation in the timetable for scrapie pathogenesis could be due to the following: dose and strain of agent; the agent donor strain, tissue, and species; route of infection; host genotype; age and sex of animal; the state of the lympho-reticular system. The tissue culture system may provide a means of dissecting specific steps or factors involved in the pathogenesis of scrapie.

Our studies clearly illustrate an increase in scrapie infectivity following exposure of NGF-treated PC12 cells to the mouse-adapted 139A agent. The unique properties of this cell line permit the study of scrapie agent replication in both dividing and non-dividing, differentiated cells. It also provides a system in which the effects of agent on specialized neuronal functions such as axonal transport and neurotransmitter activity can be analysed.

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REFERENCES


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