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PERSISTENCE OF THE SCRAPIE AGENT IN GLIAL CELLS FROM RAT GASSERIAN GANGLION

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Summary. — Persistence of the scrapie agent in glial cell monolayers from rat Gasserian ganglion have been studied by electron microscopy, virologic and histologic methods. Under chosen experimental conditions the scrapie agent had been shown to persist in cells for over 2 years. The infected cells appeared to be infectious for BALB/c mice throughout. Persistence of the scrapie agent was associated with development of a marked proliferative effect with increasing mitotic index of the cells. Changes similar to those observed in the CNS in vivo were found by means of electron microscopy. The decreased number of oncovirus-containing cells seems to indicate the decreased proportion of such cells in the culture infected with scrapie agent.

Key words: *subacute transmissible spongiform encephalopathy*; *slow virus infections*; *scrapie*; *pathogenesis*; *Gasserian ganglion cell culture*

Introduction

Scrapie agent is a typical representative of subacute transmissible spongiform encephalopathies causing slow virus infections in humans and animals. It is considered to represent a separate group of filterable infectious agents significantly different from classical viruses in a number of characteristics (Gajdusek, 1978). The interactions of STSE agents with sensitive cells are poorly studied owing to the difficulties involved with the establishment of in vitro infection. The available data have been mainly obtained in cell cultures from the brain of infected animals, whereas studies dealing with infection of intact cell cultures with STSE pathogens are scarce (Marcovits *et al.*, 1981; Cherednichenko *et al.*, 1982; Rubinstein *et al.*, 1984). In the published papers attention has been basically focused on the fact of persistence in cell cultures itself; the authors were less concentrated on the changes resulting from infection. The present paper describes the changes occurring in association with the persistence of the scrapie agent in glial culture from rat Gasserian ganglion tumour as followed by virologic, histologic and electron microscopic studies.

Materials and Methods

Scrapie agent. Scrapie agent, strain C=506 isolated in the laboratory of Dr. Gajdusek (U.S.A.) from the brain of scrapie-infected sheep and passaged in mice, was a gift of Dr. Gibbs (Institute of National Health, Bethesda, U.S.A.). The agent was maintained by passaging in mouse brain and titrated by intracerebral (i.c.) inoculation of BALB/c mice. The titre was calculated according to Reed and Muench (1938).

Cell culture. NGUK-I cells prepared from rat Gasserian ganglion tumour induced by administration of ethylnitrosourea were kindly provided by Dr. L. I. Kondakova (Laboratory of Experimental Neurooncology, Institute of Human Morphology, U.S.S.R. Academy of Medical Sciences, Moscow). NGUK-I cells mostly consist of peripheral glial cells which show pure rat karyotype, they are free of mycoplasms and contain the specific glial protein S-100. The cells were grown either in standard MEM (65%) supplemented with normal bovine serum (15%), normal calf serum (15%) and chick embryo extract (5%) or in modified MEM (70%) supplemented with normal bovine serum (30%).

Infection of cells. The cells cultured for about 1 year in standard MEM and which underwent 5 additional passages in the modified MEM have been used. A 10% brain suspension of infected mice showing symptoms of scrapie containing the agent in a titre of 6.3 log LD_{50}/0.03 ml has been used as inoculum. The inoculum was adsorbed to well-formed 2—3-day-old cell monolayers for 1 hr at 37 °C; growth medium was then added and cultivation proceeded at 37 °C with passages repeated every 3 to 8 days. A 10% suspension of normal mouse brain was used as control inoculum; intact uninfected culture served as additional control. Cultivation of infected and control cells in standard MEM (line I) for 32 passages was performed over 198 days, whereas in the modified MEM (line II) 131 passages were made over 741 days.

Cytologic studies. The materials were tested at passages 32, 51 and 99. Mitotic activity of the cells was assessed on the basis of their mitotic index. In addition, the number of pathologic mitoses and giant cells was estimated. These tests were carried out using conventional techniques (Alov *et al.*, 1974).

Electron microscopy. For electron microscopy the materials were investigated at passages 32, 51 and 130. The cells were fixed with 2% glutaraldehyde in 0.1 mol/l cacodylate buffer, additionally fixed by 2% osmium tetroxide in the same buffer, dehydrated in ascending concentrations of alcohols and embedded into Araldite-Epon mixture. For preparation of ultrathin sections, ultramicrotomes LKB-4800 or Reichert OM-3 were employed. The sections contrasted with lead citrate and saturated with uranylacetate were examined in JEM-100 B electron microscope at accelerating voltage 80 kV.

Results

To establish the presence of scrapie agent, BALB/c mice were infected by i.c. route with the cell lysates prepared by 3 freezing-thawing cycles (15 mice per group) and the infected animals were observed for at least 11 months. The development of fatal disease served as criterion for presence of the agent, the duration of the incubation period being taken into account. When cells of line I were used, bioassays were carried out from passage 4 to passage 29 at 3 to 5 passage intervals; when line II cells were used, the assays were carried out from passage 3 to passage 101 at 5 to 7 passage intervals, with prolongation of the interval at later passage levels. As shown in Fig. 1, the morbidity and death rate in infected mice were rather high, the incubation period varying from 4.5 to 12 months. The duration of the incubation period was independent of the passage level and was generally equal to 9 or 10 months. The infectivity of cells varied in the course of passages remaining high up to late passage levels in both cell lines.

The microscopy of cell cultures revealed a marked proliferative effect starting from passages 4 to 7, i.e. from days 12 to 34 of cultivation. This

was evident from more rapid formation of the cell monolayer and from its higher density as compared to either intact cells or cells inoculated with normal mouse brain suspension, at the same passage level. In the line I cells the proliferative effect was marked at passages 4, 12, 18, 20, 21, 25 and 28, i.e. it persisted up to day 173 of incubation. Direct cell counts at passage level 20 demonstrated that the number of cells per flask in the scrapie--infected culture was 3 times higher than in intact cells or than in the culture inoculated with normal mouse brain suspension. In line II cells the proliferative effect appeared at passage 7, was registered at passages 19 and 29 and remained marked up to passage 130, i. e. on the day 741 of observation. In latter cells at passages 32, 51 and 99 the mitotic index, the number of pathologic mitoses and the number of giant cells were determined in the course of culture growth from incubation days 1 to 4. The indices were calculated comparing to the respective data from experimental and control cultures. At passage 32 the mitotic index was in infected culture 1.7 times higher than in control, the number of pathologic mitoses increased 1.5 times and the number of giant cells 4 times. The increasing tendency of mitotic activity was still observed at passage 51; mitotic index in the test group was 2.8 times higher than in corresponding controls, the incidence of pathologic mitoses being unchanged and the number of giant cells being elevated 4 times. At passage 99 the scrapie-agent-infected cells still had an increased mitotic activity: mitotic index was 4.8 times higher than in control cultures. This increase of mitotic activity occurred during logarithmic phase of culture growth. Essentially, no difference between the levels of pathologic mitoses

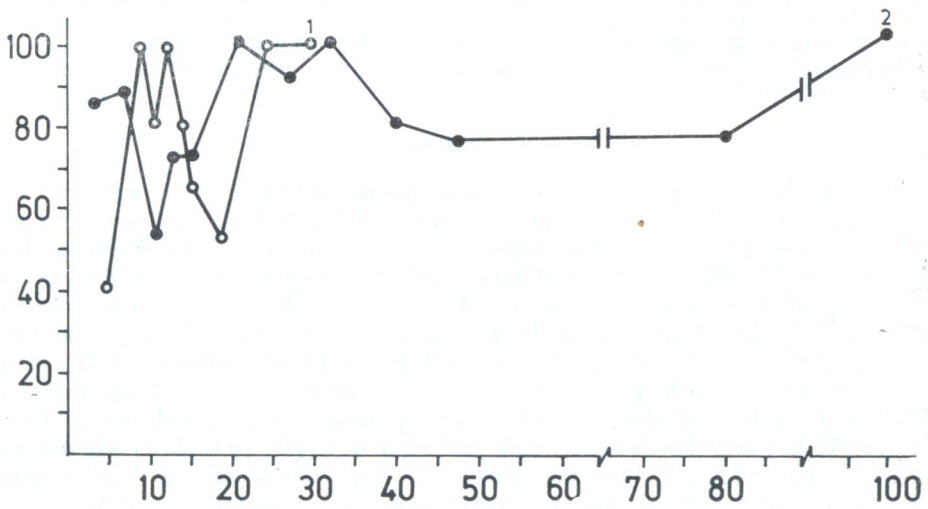

Fig. 1.
Infectivity of NGUK-1 cells infected with scrapie agent for BALB/c mice
Abscissa: the number of passages; ordinate: deaths of mice infected with cell lysates (%).
1 — cell line I; 2 — cell line II.

Table 1. The influence of scrapie agent persistence on the content of oncovirus particles in the NGUK-I cells

Cells	Total number of sections	Number of oncovirus-free cell sections	
		absolute number	%
Intact	108	10	9
Inoculated with control brain suspension	124	2	1.6
Infected with the scrapie agent	117	45	38

and the number of giant cells was observed in the compared groups at passage 99.

Electron microscopy of control and test cultures has revealed the presence of some submicroscopic changes in infected cells. The cytoplasm of latter contained so-called myeline-like structures and significantly more giant lysosome-like formations (Fig. 2). Proliferation and hypertrophy of the Golgi complex was invariably observed. A marked margination of chromatin was also seen. Intensive vacuolization of infected cells, in some cases limited to the ectoplasmic zone, was one of the characteristic features (Fig. 3-I). Vacuolization was basically related to the swelling of vesicles and channels of rough endoplasmic reticulum, as well as of cisterns and vacuoles of the Golgi complex (Fig. 3-II). The above mentioned changes were seen at passage 32 and could still be registered as late as at passage 130.

Ultrastructural studies suggested a decrease in the number of scrapie-agent-infected cells in which oncornavirus particles, that are constantly present in NGUK-I cells, were found. This encouraged a more thorough quantitation of oncovirus particles in ultrathin sections. From each group of cells at passage level 32 (intact cells, cells inoculated by normal mouse brain suspension, cells infected with the scrapie agent) over 100 at random chosen cell sections were examined and the number of sections containing and not containing oncovirus particles was counted. Five to ten blocks of cells from parallel flasks were taken from each group for sampling. As seen from Table 1, 9% and 1.6% of sections without oncoviruses were found in intact cells and in the cells inoculated by normal mouse brain, respectively. In contrast, among cells persistently infected with the scrapie agent, the number of such sections amounted to 38%. This pointed to a reduction of the number of infected cells containing oncovirus particles, which seems to be related to a decrease of oncovirus content in scrapie-agent-infected cells.

Discussion

The study of the interactions of STSE agent with sensitive target cells is a promising trend in the investigation of their pathogenesis. The findings obtained from in vivo experiments allowed to show some peculiarities of the

spread of the agent in the organism and to determine their location in the organs. Studies in vitro on interaction between the agent and sensitive cells in the course of prolonged persistence typical of STSE is very important for understanding of the pathogenesis of disease. These experimental conditions have been designed in the present paper, in which prolonged persistence of the scrapie agent has been established. It should be noted that the ability of STSE pathogens to persist over a certain period in vitro has been demonstrated during the cultivation of brain cells from infected animals (Gustafson and Kanitz, 1965; Clarke and Heig, 1970; Heig, 1970; Gajdusek *et al.*, 1972). However, this method did not allow to analyse the fate of the agent or its effect on the cells in the course of infection. Therefore, the attempts to infect cells by STSE agents and their subsequent study seemed more instructive (Asher *et al.*, 1979; Yanagihara *et al.*, 1980; Cherednichenko *et al.*, 1982). Unlike the authors who have obtained scrapie-agent infection by additional treatment of cells with lysolecithin, DEAE dextrane or lidase (Clarke and Millson, 1976; Cherednichenko *et al.*, 1982) we managed to infect glial cells without pretreatment and to reproduce prolonged persistence of the scrapie agent throughout a 2-year period. The fact of persistence itself was confirmed by regular observations of infectivity of the cultures up to passage 100; average duration of incubation period in mice infected with cultural material was 9 or 10 months, which is consistent with the duration of the incubation period at low infectious dose. It should be noted that long-term persistence of the agent failed to elicit a cytolytic effect; on the contrary, starting from passages 4 to 7 a marked proliferative effect developed. Meanwhile, cellular mitotic index was increased and kept elevated throughout the period of persistence. At passage 32, the mitotic index in infected cells was 1.7 times higher than in control, at passages 51 and 99 it became 2.8 times, and 4.8 times higher, respectively. This was associated with a certain increase in the number of pathologic mitoses and in the production of giant cells which may be an indirect evidence for the agent's persistence. According to our data, brain organ cultures of mice infected with the scrapie agent showed a greater tendency for replication as compared to control cultures, which was also observed by other authors during cultivation of brain explants of scrapie-infected mice (Field and Windsor, 1965; Caspary and Bell, 1971). It is noteworthy in this connection that at early stages of in vivo infection the scrapie agent caused hypertrophy and increase in the amount of nuclear protein in neurons of the IIIrd cortical layer (Roikhel *et al.*, 1983).

It is possible that proliferative action of the scrapie agent characterizes the peculiarity of the interaction of this pathogen with glial cells which has been also demonstrated by Marcovits and coworkers (1982, 1983), in contrast to persistent infection of the agent in L-cells in which the growth of infected cells appeared to be slowed down and their karyologic characteristics were altered (Cherednichenko *et al.*, 1982; Mikhailova and Cherednichenko, 1984). It can be suggested that the capacity of scrapie agent to persist in glial cells, causing certain changes in them, points to a probable contribution of these

cells for pathogenesis of STSE infection in vivo, which was also stressed by Heig and Clarke (1971). Our electron microscopic findings also provide indirect evidence for this suggestion. Of interest in this respect, are marked metamorphosis and alteration of membrane elements observed in infected cells characteristic for the pathologic process of STSE in vivo.

During cultivation of STSE agents in vitro peculiar inability to interfere with reproduction of some classic viruses has been demonstrated (Nemo *et al.*, 1980). We have also shown that infection of mice with the scrapie agent failed to change the level of accumulation of superinfecting tick-borne encephalitis virus in the brain. However, the data presented in this paper indicate that long-term persistence of scrapie agent in glial cells leads to interference with accumulation of retroviruses. Marcovits and coworkers (1983) have reported the tendency to decrease of the number of A-type particles in scrapie-infected cells. We believe that competition for membrane structures due to close association of STSE agents with cellular membranes is one of the causes of observed interference with oncovirus accumulation. Therefore, the available data indicating that STSE agent-infected cells acquire neoplastic characteristics and the ability to induce the production of tumours in nude mice seem to be of interest (Manuelidis *et al.*, 1983; Manuelidis, 1985).

Further studies on the behavior of STSE agents in vitro is a likely source of new data for understanding interactions of these poorly studied agents with target cells.

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Explanation to Micrographs (Plates V—VI):

Fig. 2. Submicroscopic features of glial cells infected with the scrapie agent.
I — myeline-like and lysosome-like structures. Magn. × 42 000
II — large lysosome-like formation. Magn. × 50 000

Fig. 3. Large vacuolized areas in the cytoplasm of glial cells infected with the scrapie agent
I — vacuolization in the cytoplasmic zone. Magn. × 11 000
II — vacuolization mainly related to the swelling of vesicles and channels of endoplasmic reticulum. Magn. × 16 800