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STUDY OF EARLY STAGES OF THE PATHOGENESIS OF SCRAPIE IN EXPERIMENTALLY INFECTED MICE

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Summary. — Virological, histological, and electron microscopic methods were used to study the early manifestations of infection in brains and spleens of mice experimentally infected with the scrapie agent. A statistically significant increase in the spleen weight was demonstrated during the incubation period; this occurred only after intracerebral (i.c.) inoculation of the scrapie-containing brain suspensions and subuctaneous (s.c.) inoculation of the scrapie-containing spleen suspensions. No differences were observed between the scrapie agents replicating in the brain or spleen. In the brain at early stages of the incubation period, ultrastructural changes were observed in pre- and post-synaptic areas. The importance of these findings for understanding of the pathogenesis of subacute transmissible spongiform encephalopathy (STSE) is discussed.

Key words: *subacute transmissible spongiform encephalopathy*; *scrapie agent*; *pathogenesis*

Introduction

Scrapie is a slow progressive disease of the CNS of sheep belonging to the group of STSE which are caused by infectious agents possessing some properties of the classical conventional viruses and at the same time markedly differing from them in a number of characteristics (Gajdusek, 1978). The STSE group includes both animal (scrapie and transmissible mink encephalopathy) and man diseases (kuru and Creutzfeldt-Jacob disease); suggestions were made to include into the group of slow infections also a number of chronic diseases of the CNS in man with unknown etiology (Gajdusek and Gibbs, 1977). The infection caused by the scrapie agent adapted to mice is considered to be a model system for study of the features of pathogenesis of other slow infections of CNS in man and animals (Kimberlin, 1976*a*). As the virological diagnosis of scrapie is complicated and time-consuming, it seems expedient to investigate thoroughly the early stages of the incubation period in those organs (brain and spleen) in which the agent accumulates more intensively during the clinical stage of the disease. The interest to

study the early lesions in target organs is also due to the lack of definite concepts on the sequence and relationships of phases in the scrapie pathogenesis (Kimberlin, 1976*b*). The present work was aimed at the study of morphological lesions in the brain and spleen of the inoculated animals and of some properties of the agent accumulating in these organs in the early stages of the incubation period.

Materials and Methods

Animals. BALB/c mice 23-week-old were used.

The agent and methods of infection. Scrapie agent, strain C-506, was isolated in Dr. D. C. Gajdusek's laboratory (Bethesda, MD) from the brain of a sheep with scrapie; it subsequently underwent 4 passages in mice as obtained from Dr. N. V. Loginova (D. I. Ivanovsky Institute of Virology, Moscow). The strain was maintained by i.c. inoculation of BALB/c mice with 10% suspensions of the organs under study in physiological saline, using 0.03 ml inocula of the virus-containing suspensions for i.c. inoculations and 0.25 ml for s.c. inoculations. The results of the inoculations were assessed by the number of animals developing a typical clinical picture and histological lesions, as well as the duration of the incubation period. The inoculated animals were observed for 11 months.

Histological studies. The organs were fixed in 10% formalin and embedded into paraffin; sections were stained either with hematoxylin-eosin or according to the methods of Nissl and Cajal.

Electron microscopy. The material was fixed in 2% glutaraldehyde in cacodylate buffer, pH 7.2, followed by fixation with 2% OsO_4 solution in the same buffer, dehydrated in an ascending series of alcohols and embedded to Epon-Araldite. Ultrathin sections were cut in either LKB-4800 or Reichert ultramicrotomes, contrasted with uranyl acetate and lead citrate and examined in a JEM-100 electron microscope at an accelerating voltage of 80 kV.

The results were statistically evaluated using Student's criterion for the assessment of validity.

Results

Mice were inoculated i.c. or s.c. with a 10% scrapie-containing suspension of mice brain or spleen, using 10 animals per group. The titre of the agent in the inocula was 5.4 to 5.7 LD_{50}/0.03 ml. As controls, mice of the same batch were inoculated with 10% suspensions of the brain or spleen from normal non-infected mice. On day 40 post inoculation, the animals without clinical manifestations of the disease were sacrificed, and their brains and spleens were used for virological, histological, and electron microscopic examinations. When the weight of the brains was compared, no differences between experimental and control groups were observed; a statistically significant increase in the weights of infected mice spleens was demonstrated, however. It may be seen in Table 1 that this occurred only after i.c. inoculation of the scrapie-containing brain suspensions and s.c. inoculation of the scrapie-containing spleen suspensions. Histological examinations of spleens of these mice revealed hyperplasia of the lymphoid tissue. Inoculation of normal organ suspensions let to no increase in the spleen weights.

For detection of the scrapie agent in the brains and spleens of the inoculated animals sacrificed at 40 days, mice were inoculated i.c. and s.c. with the same substrate. As may be seen in Table 2, no agent was found in the brains of mice originally inoculated s.c. with a spleen or brain agent-containing suspension, the agent being found in the spleens of these mice, however.

Table 1. Spleen weights in mice 40 days after inoculation with the scrapie agent in relation to the agent-containing substrate and inoculation route

The agent-containing substrate	Inoculation route	Spleen weight (mg)		P
		Control	Experiment	
Brain	i.c.	167 ± 13	321 ± 52	$0.95 < P < 0.9$
Brain	s.c.	227 ± 31	217 ± 21	$P < 0.95$
Spleen	i.c.	202 ± 21	234 ± 36	$P < 0.95$
Spleen	s.c.	220 ± 19	327 ± 29	$P > 0.99$

After initial i.c. inoculation of spleen suspensions the agent was detected in both the spleen and brain by either inoculation route. The duration of the incubation period with the same material was shorter after i.c. than after s.c. inoculation (144—175 and 185—215 days, respectively).

Histological examinations of the brains and spleens of mice sacrificed early and at 40 days post inoculation showed no differences between experimental and control groups. Electron microscopic examinations of the brains from these mice revealed changes absent in the corresponding preparations of the control groups. The cerebellum showed marked myelin destruction manifested by focal swelling of axonal myelin sheaths and disintegration of spiral myelin plates (Fig. 1-II). There was degeneration of synapses mostly of the "clear type" accompanied by oedema of the presynaptic terminal and clearing of the cytoplasm. In some synapses, the number of presynaptic vesicles was decreased or their fusion was observed (Fig. 1-II). Sometimes, swelling and rounding of postsynaptic terminals (Fig. 1-III, -IV) as well as swelling of astrocytic processes and transparency

Table 2. Detection of the scrapie agent in brain and spleen of mice in relation to the kind of scrapie-containing substrate and inoculation route

Scrapie-containing substrate and inoculation route	Organ examined 40 days post-inoculation	Route of inoculation of organ suspension for agent detection	Mortality	Incubation period (days)
Brain, s.c.	Brain	i.c.	0/10*	—
		s.c.	n.d.	—
	Spleen	i.c.	12/12	148
		s.c.	9/9	185
Spleen, i.c.	Brain	i.c.	10/10	175
		s.c.	n.d.	—
	Spleen	i.c.	12/12	144
		s.c.	10/10	188
Spleen, s.c.	Brain	i.c.	0/11	—
		s.c.	0/13	—
	Spleen	i.c.	10/10	173
		s.c.	12/13	215

* Numerator — number of dead animals, denominator — number of inoculated animals.
n.d. = not done

of their cytoplasm could be seen. Changes in the neurons, microglial cells and capillary endothelium did not virtually differ from controls, although here and there degenerated mitochondria (Fig. 1-I) were found in the cytoplasm of these cells. The specificity of the changes observed is demonstrated in Table 3; it may be seen that unlike to mitochondria, the lesions in synapses and myelin degeneration were marked in the experimental groups only; in controls they were either absent or poor and found in single cells.

Some animals were kept for 5—7 months until the development of marked clinical signs (exhaustion,round movements, hunched backs, haemorrhagies in the tails). Histological examinations of the brains of these mice revealed the presence of spongiosis in the cerebral cortex (Fig. 2-I), Ammon's horn (Fig. 2-II) and subcortical nuclei, as well as loss of neurons in the cerebral cortex and subcortical nuclei (Fig. 3-I). In such foci there was marked astrocytic proliferation with hypertrophy of cell bodies and processes (Fig. 3-II). Marked cytoplasmic vacuolization was frequently observed in cerebral cortex neurons, cerebellar nuclei, and subcortical area. Electron microscopic examinations of the brains of mice sacrificed in the clinical stage of the disease revealed the presence of vacuole-like formations of different sizes in the neuropil (Fig. 4-I). These formations represented swollen areas of the cytoplasm of astrocytes or their processes, in part they represented fragments of swollen and rounded dendrites or dendrite terminals (Fig. 1-III, 1-IV). Sometimes, accumulations of characteristic membrane formations could be seen in the lumen of vacuoles or in intercellular spaces (Fig. 4-II). Cytoplasmic vacuolization was frequently observed in cell elements. In neurons, vacuoles formed at the expense of swollen parts of the endoplasmic reticulum, there was focal degeneration of mitochondria, hyperplasia of filamentous structures, and marked lysosomal reaction. In the cytoplasm of Purkinje cells, astrocytes, and oligodendrocytes there were lysosome-like intracytoplasmic inclusions

Table 3. The presence of early morphological lesions in the CNS of mice 40 days after inoculation with suspensions of normal and scrapie-containing organs

Inoculum and inoculation route		Lesions in the CNS		
		myelin degeneration	destruction of synapses	local changes in mitochondria
Normal spleen,	i.c.	−	±	±
	s.c.	−	−	−
Normal brain,	i.c.	±	−	±
	s.c.	−	−	−
Scrapie-containing spleen	i.c.	+++	++	+
	s.c.	+++	++	±
Scrapie-containing brain	i.c.	++	++	±
	s.c.	++	+	±

Notice: − = no lesions, ± = lesions in occasional cell elements of the CNS, + = lesions in less than 50%, ++ = approximately in 50%, +++ = more than in 50% of cell elements of the CNS.

varying in shape and structure and resembling myelin figures. Changes in the myelinized axons ranged from disintegration of spiral myelin plates surrounding the intact axoplasm to vacuolization of axoplasm and occurrence of pleomorphic inclusions with intact myelin sheath around the axon. Change in the synapses corresponded to those described in the CNS of mice sacrificed in the incubation period, but were more marked.

Discussion

In the present study we looked for early changes in the target organs (brain and spleen) of mice which are the sites of the predominant multiplication of the scrapie agent (Eklund *et al.*, 1965; Zlotnik, 1970). In the design of our experiments we proceeded from the assumption of Fraser and Dickinson (1967) of a possible modification of the scrapie agent upon its accumulation in the brain and spleen. To achieve better manifestation of the presumed tissue tropism we used suspensions of the spleen and brain as different agent-containing substrates inoculated i.c. or s.c., modelling the conditions of "homology" or "heterology". According to the test of the spleen weight increase as an early response to the agent, positive results were observed only with the "homologous" agent-containing inoculum and inoculation route (Table 1). The assumption of the existence of spleen and brain variants of the agent was not confirmed under selective conditions for the manifestation of such variants tested by criteria as infected mice mortality and duration of the incubation period (Table 2); this indirectly corresponds to the data by Outram (1976). At the same time, one gets an impression that the "homology" of the agent-containing substrate and inoculation route are important for the phenomenon of the spleen weight increase. This question requires further study. Our data have also shown the previously described (Dickinson and Fraser, 1972) shortening of the incubation period after i.c. inoculation as compared to the peripheral route occurred when-both scrapie-containing suspensions from brain and spleen were used as inocula.

Morphological examinations of brains of the mice sacrificed 40 days postinoculation by light microscopy showed no differences between the experimental and control groups in accordance with available reports (Dubois-Dalcq *et al.*, 1977). Astrocyte hypertrophy described as an early sign was observed at later stages of the incubation period (Field, 1967; Pattison and Jones, 1967). At the same time, we observed in the CNS of the mice sacrificed in the clinical period status spongiosus, loss of neurons and astrocytic hypertrophy which testified to the specific nature of the pathological process typical of STSE (Beck and Daniel, 1965; Chandler, 1961). Electron microscopy of the CNS of mice early in the infection showed certain changes while lights microscopy revealed no changes at this stage of infection.

Of special interest were the lesions we observed in synapses and presynaptic vesicles containing chemical mediators facilitating transmission of excitation (Gray, 1967). These changes seem to be the morphological basis of behaviorial and motor activity disorders of scrapie-infected mice during

the incubation period shown by some authors (Cathala *et al.*, 1980; Heitzman and Corp, 1968; McFarland and Hotchin, 1980; Savage and Field, 1965; Suckling *et al.*, 1976). It should also be noted that changes in the synapses were alike to those observed in the CNS of mice sacrificed with clinical disease which supplements the picture of ultrastructural changes described by others (Baringer and Prusiner, 1978; Chandler, 1967; Lampert *et al.*, 1971). Possibly the changes observed in the early stages of infection in axon and dendrite terminals which are components of synapses may be considered to be the early stages of status spongiosus formation.

It was of interest that the changes in the CNS detected during the incubation period were observed both in the presence and in the absence of detectable scrapie agent. In the latter instance the intensity of the changes was lower. Chandler (1967, 1968, 1969) observed some changes in the CNS of rats and mice in the incubation period of scrapie, but those lesions were observed at later intervals. The results of this study indicate that in slow infections caused by STSE agents there occurs an early appearance and gradual development of morphological lesions in the CNS detectable at submicroscopic levels and underlying the functional disorders and clinical symptoms.

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Explanation of Micrographs (Plates XVI—XIX):

Fig. 1. Mouse brain in the pre-clinical stage of scrapie 40 days after inoculation.
I — cerebellum, degeneration of mitochondtria × 27.000;
II — cerebral cortex, disintegration of myelin in a myelinized axon and fusion of synaptic vesicles in the synapse. × 27,500;
III — cerebral cortex, early stage of swelling of the postsynaptic terminal. × 27,500;
IV — cerebellum, marked swelling of the postsynaptic terminal. × 27,500.
Figs. 2—3. Histological lesions in the brain of mice with clinical signs of scrapie.
2-I — spongious oedema in the cerebral cortex, hematoxylin-eosin staining. × 200;
2-II — spongious oedema in the Ammon's horn, hematoxylin-eosin staining. × 200;
3-I — loss of neurons in a subcortical ganglion, Nissl's stain. × 100;
3-II — hyperplasia and hypertrophy of astrocytes in the cerebral cortex, Cajal's stain. × 200.
Fig. 4. Ultrastructural changes in the brains of mice with clinical scrapie.
I — Vacuoles of different sizes (status spongiosus) in the cerebral cortex. × 14,750;
II — typical membranous formations in cerebellum × 30,000.