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Transmissible Mink Encephalopathy: Studies on the Peripheral Lymphocyte

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Circulating lymphocytes from mink with transmissible mink encephalopathy were studied for the presence of virus particles and for infectivity. Electron microscope examination of phytohemagglutinin-stimulated lymphocyte cultures revealed no evidence of virus-like particles. Circulating lymphocytes were also found to be noninfectious when the transmissible agent could be demonstrated in intact lymphoid organs in infected mink. A lymphopenia was detected in affected mink during the late clinical stage of disease, but this was most likely a secondary effect due to a terminal stress factor.

Transmissible mink encephalopathy (TME) is a slow virus disease belonging to the group of transmissible spongiform polioencephalopathies. Studies on mink susceptibility to sheep scrapie have indicated that TME is a form of scrapie resulting from the natural exposure of mink to infected sheep tissues (9).

One of the more interesting observations made on scrapie infection in mice is that the scrapie agent appears to replicate in the reticuloendothelial system (RES) relatively early during the long incubation period (6). The concept of RES replication as an important step in the pathogenesis of scrapie has been reenforced by the observation of significantly longer incubation periods in splenectomized animals inoculated by the intraperitoneal route (2, 7). The question therefore arises as to which of the cell populations in the RES are involved in this extraneural phase of infection. It has been hypothesized that the lymphocyte may be infected and may serve to disseminate the infection throughout the body (6). Infection of the lymphocyte may also be responsible for the lack of a detectable specific immune response in either scrapie (8) or TME (12). The following experiments were designed to study peripheral lymphocytes in TME-infected mink and to determine their infectivity in relationship to intact lymphoid organs.

MATERIALS AND METHODS

The experiment was divided into two parts, each part performed concurrently. The first part of the experiment involved the separation of blood constituents from mink in the late clinical stages of encephalopathy. Each blood fraction was assayed for infectivity by mink inoculation. Lymphocytes were assayed from blood and after culture with or without phytohemmaglutinin (PHA) stimulation.

Blood fractionation. Four TME-affected mink were anesthetized with ether and exsanquinated by intracardiac puncture. Blood was collected into bottles containing 1 mg of disodium ethylenediaminetetraacetate per ml and centrifuged at 1,000 × *g* for 30 min. The plasma was removed and the buffy coat was resuspended in a small amount of plasma. The mononuclear leukocytes were separated by using a modification of the silicone flotation method of Carper (1). For this procedure a mixture composed of equal parts of silicone (Dow Corning Corp., Midland, Mich.) fluids 550 and 710 was found to produce the best separation of mononuclear cells from polymorphonuclear leukocytes and red blood cells. After centrifugation at 1,700 × *g* for 30 min, the cells remaining above the silicone were resuspended in Eagle minimal essential medium modified for suspension cultures (4). Platelets were separated from the mononuclear leukocytes by centrifugation at 400 × *g* for 10 min. The final yield from 100 ml of whole blood was 7.8×10^7 cells, of which 94% were lymphocytes by differential count.

Lymphocyte cultivation. A concentration of 1.5×10^7 cells was added to tightly stoppered prescription bottles containing 15 ml of modified Eagle medium supplemented with 20% heat-inactivated fetal calf serum, 100 U of penicillin, and 100 μg of streptomycin per ml. In addition, Phytohemagglutinin-M (Difco) was added to some of the cultures at a final concentration of 2%. After 45 h of incubation at 37 C, the cells were centrifuged at 1,000 × *g* for 10 min. The pellets and supernatant fluids were collected for mink inoculation and examination by electron microscopy.

Electron microscopy. Only lymphocytes cultured in the presence of PHA were examined. The pellet containing 4.0×10^7 cells was resuspended

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in a few milliliters of calf serum and again centrifuged. The serum was removed and 5 ml of 3.5% glutaraldehyde in 0.1 M cacodylate buffer (ph 7.2) was carefully layered over the cell pellet. After overnight fixation in a stoppered tube at 4 C, the pellet was cut into cubes (1 by 2 mm) and washed in cacodylate buffer. Specimens were postfixed in 1% osmium tetroxide for 1 h, dehydrated, and embedded in Mollenhauer epoxy resin mixture (15). Thin sections were cut with glass knives, stained in 25% uranyl acetate for 20 to 30 min and lead citrate for 2 to 5 min, and then examined with a Hitachi HU-11B2 electron microscope.

In the second part of the experiment, four mink were each inoculated intracerebrally with 10^5 mean lethal dose of the TME agent, and two were similarly inoculated with normal mink brain. At weekly intervals, two TME-inoculated mink and one control were anesthetized with ether, and 6 ml of blood was withdrawn by intracardiac puncture. Each mink in the experiment was therefore bled every 2 weeks. Lymphocytes from the TME-inoculated animals were concentrated by the silicone flotation method previously described and assayed for infectivity by mink inoculation.

RESULTS

Infectivity of blood cells and fluids from mink in the late clinical stages of TME. Whole blood, plasma, red blood cells (10%, washed suspension), platelets (10%, washed suspension), white blood cells (1.7×10^7/ml), cultured lymphocytes (1.5×10^7/ml), supernatant fluids from cultured lymphocytes, cultured lymphocytes simulated by PHA (1.5×10^7/ml), and the supernatant fluids from the stimulated cultures were intracerebrally inoculated in 0.1-ml quantities into two mink each. After a 1-year period of observation, none of the 18 inoculated mink were observed to develop clinical signs of TME, and pathological examination of at least one animal from each group revealed no lesions of a spongiform encephalopathy.

Examination of PHA-stimulated lymphocytes for virus. After 45 h of culture in the presence of PHA, all surviving cells appeared to be lymphocytes, most of which had undergone a transformation to a more lymphoblastic appearance than those observed in the unstimulated cultures. Electron microscope examination of these cells revealed no evidence of the presence of virus or virus-like particles.

Lymphocyte counts and infectivity during the incubation period. Lymphocyte counts for the four TME-inoculated and two control mink are plotted in Fig. 1. Two mink inoculated with the TME agent died during the course of the experiment as a result of the intracardiac bleeding procedure. Brain, spleen, mesenteric lymph node, liver, and kidney tissues from these animals were tested for infectivity by mink inoculation (Tables 1 and 2). One of the two remaining TME-inoculated mink developed clinical disease 18 weeks after infection. This animal became progressively more ataxic but was in good body condition when it died during the 22nd week from ether anesthesia. The second mink developed signs of incoordination 20 weeks after inoculation. This animal had a lymphocyte count of 8,905/mm^3 on the 22nd week and 805/mm^3 2 weeks later when in a completely debilitated, moribund state.

Lymphocytes from the TME-inoculated mink were evaluated for infectivity at various times in the post-inoculation period. Samples from weeks 1–5, 7, 9, 10, 12, 14, 16–17 (pooled), 19, and 22 were each intracerebrally inoculated (0.1 ml) into two mink. Each weekly specimen represented

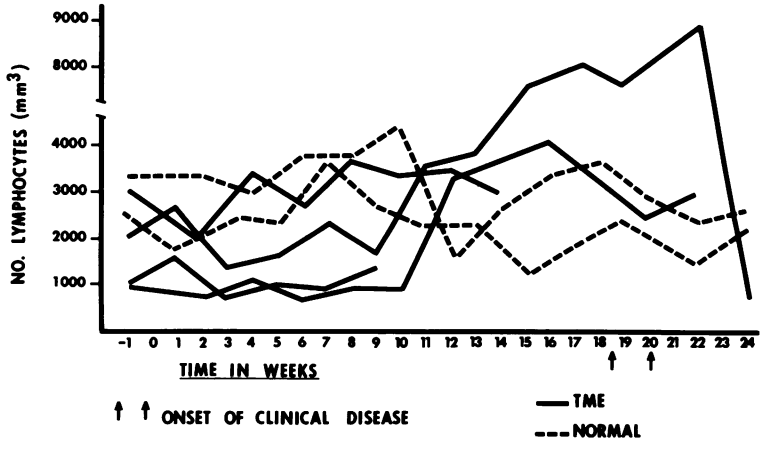

FIG. 1. *Lymphocyte counts in mink inoculated intracerebrally with normal mink brain or brain containing the transmissible mink encephalopathy agent.*

TABLE 1. *Infectivity of tissues from a mink which died 9 weeks after intracerebral inoculation with the transmissible mink encephalopathy agent*

Tissue (10% wt/vol suspension)	Infectivity[a]
Brain	2/2 (23, 23)
Spleen	2/2 (28, 29)
Mesenteric lymph node	2/2 (28, 31)
Liver	0/2
Kidney	0/2

[a] Animals inoculated intracerebrally with 0.1 ml of tissue suspension which develops TME within 1 year of inoculation/number inoculated. Incubation periods in weeks are in parentheses.

TABLE 2. *Infectivity of tissues from a mink which died 14 weeks after intracerebral inoculation with the transmissible mink encephalopathy agent*

Tissue (10% wt/vol suspension)	Infectivity[a]
Brain	2/2 (24, 25)
Spleen	2/2 (34, 42)
Mesenteric lymph node	2/2 (23, 26)
Liver	0/2
Kidney	0/2

[a] Animals inoculated intracerebrally with 0.1 ml of tissue suspension which develops TME within 1 year of inoculation/number inoculated. Incubation periods in weeks are in parentheses.

pooled lymphocytes from two mink which had been diluted to a final concentration of 2×10^6/0.1 ml. After a 1-year observation period, none of the lymphocyte inocula were found to produce clinical or pathological evidence of disease.

Lymphocyte counts during clinical disease. To further examine the precipitous drop in circulating lymphocytes observed in one animal in the previous experiment, lymphocyte counts were followed in 10 mink during the clinical course of disease. These animals were all orally inoculated with either TME mink brain or spleen suspensions. Their clinical disease was divided into three stages. The first stage represented early behavioral changes and the presence of beginning locomotor incoordination. Severe incoordination and somnolence were the criteria for stage two, and stage three was complete debilitation with little or no outward response to external stimuli. Stage three was usually accompanied by a reduction in body temperature and a rise in hematocrit and preceded death by only a few hours. It was not possible to obtain counts from every animal in all three stages (Table 3); however, there was a consistent reduction in circulating lymphocytes in all animals examined in stage three.

TABLE 3. *Lymphocyte counts in mink during the clinical course of encephalopathy*

Mink (no.)	Sex	Lymphocytes (differential %/no. per mm³)		
		Stage 1	Stage 2	Stage 3
240	M	46/1,973	44/3,001	10/715
239	F	NA[a]	27/1,960	21/1,455
241	M	53/1,603	42/1,802	8/945
223	F	42/1,155	39/1,104	6/1,188
230	F	32/3,807	48/3,062	14/847
252	M	64/2,464	46/1,973	13/645
250	M	40/3,392	36/1,663	6/815
247	M	31/1,296	57/6,207	NA
228	M	71/3,280	60/2,772	NA
249	M	35/1,425	34/1,494	NA

[a] NA, data not available.

DISCUSSION

In previous experiments with TME, sera from terminally affected mink inoculated either intramuscularly or intracerebrally were found to be noninfectious (11). The present experiments expand on these findings by further studying blood cell fractions. Intracerebral inoculation of mink has proven to be a relatively sensitive measure of the TME agent, detecting infectivity in as little as a 0.1-ml quantity of a $10^{-7.5}$ dilution of brain (11). The failure of an inoculum containing 2×10^6 lymphocytes to produce TME would indicate that few, if any, of these cells are infected.

Pooled lymphocytes from four TME-affected mink were cultured in the presence of PHA in an attempt to augment the detection of virus in these cells. Enhancement of viral replication in PHA-stimulated human leukocyte cultures has been shown for herpes simplex virus (16), mumps virus (3), vesicular stomatitis virus (5), and vaccinia (13). It has also been shown that PHA cultivation of lymphocytes from cattle with bovine lymphosarcoma induces the formation of a C-type virus (14). Lymphocytes from TME-affected mink cultured in the presence of PHA were neither infectious nor were any virus-like particles detected by electron microscope examination.

Lymphocytes from mink inoculated intracerebrally with the TME agent were also noninfectious when tested at weekly or biweekly intervals during the incubation period. An intracerebral route of inoculation was used because it gives shorter and more uniform incubation periods (11). One possible hazard to this procedure is that intracerebral inoculation may by-

pass the lymphoid phase of replication. However, this does not seem to have been the case since spleen and mesenteric lymph node were found to contain the TME agent as early as 9 weeks after inoculation.

Lymphopenia was observed in the terminal stage of TME. Whereas this was usually both a relative and absolute lymphopenia, the former was found to be a more accurate indicator because of the phenomenon of hemoconcentration. Mink severely affected with TME are incapacitated to a degree which interferes with their normal food and water intake, and death usually results from inanition. Shock and sludging of the blood vessels might, therefore, explain a drop in circulating lymphocytes. However, since this effect was seen only on lymphocytes and not on neutrophils, a terminal stress factor may also be responsible. This could be further examined by measuring corticosteroid levels in terminally affected animals.

The most important observation made in this experiment is that mature, circulating lymphocytes were noninfectious during a time when the TME agent was demonstrable in intact lymphoid organs. It is still possible that the TME agent may replicate only in young, immature lymphocytes which are not released into the circulation. If this were true, and if the TME agent is absent or "masked" in mature lymphocytes, reversion to a more lymphoblastic state by PHA stimulation does not appear to "unmask" infectivity. Lymphoblasts have been found to have a high specific infectivity in mouse scrapie (10).

In transmissible mink encephalopathy there appear to be no morphologic or antigenic indicators of infection outside the central nervous system. This places severe restrictions on pathogenesis studies when only a single criteria, infectivity, can be used to judge cell susceptibility. In light of these limitations, it may be unwise at this time to completely dismiss the circulating lymphocyte from playing any role in the replication and dissemination of the transmissible agent of mink encephalopathy. But it is now possible to pursue alternative explanations more vigorously. The fixed macrophage may serve as an initial point for the uptake and replication of the agent. However, experiments in RES blockade, in which mice were pretreated with fatty acid esters (ethyl stearate, methyl palmitate) before intraperitoneal inoculation of the scrapie agent, failed to produce an appreciable alteration in the disease (unpublished data). It is also possible that the agent may replicate and migrate within neural components in lymphoid organs, with the RES acting only as a point of concentration due to its function in removing foreign substances from blood and lymph circulation.

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