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Creutzfeldt-Jakob Disease in Mice: Persistent Viremia and Preferential Replication of Virus in Low-Density Lymphocytes

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The mode of replication of the "unconventional virus" of Creutzfeldt-Jakob disease was studied in BALB/c mice infected intracerebrally. Virus was detected in the brain, spleen, lung, thymus, liver, kidney, and blood, but not in urine, at various time intervals after inoculation. The highest infectivity was present in the spleen from the second through the ninth weeks postinfection. Density gradient separation of spleen cells with colloidal silica (Percoll) revealed that the highest concentration of virus was present in blastoid cells from lower-density (1.05 to 1.07 g/ml) fractions. These results suggest that blastoid cells play an important role as the initial replication site of virus in the pathogenesis of Creutzfeldt-Jakob disease in mice.

It is now well established that Creutzfeldt-Jakob disease (CJD) is an "unconventional" virus-induced slow infection of the central nervous system (CNS) of humans and is classified as one of the naturally occurring "subacute spongiform virus encephalopathies" (9). The pathology of these encephalopathies is not associated with any evidence of host reaction of an inflammatory or immunological type, and virus-specific antibodies have never been detected.

Although structural changes are limited to the CNS, virus can be recovered not only from CNS but also from several extraneural tissues (1, 6, 13, 21, 22, 24, 28). For example, in experimental animals, the virus was present in the CNS, spleen, lymph node, thymus, kidney, liver, cerebrospinal fluid, and peripheral blood (1, 13, 28). The study reported here was undertaken to obtain data on the mode of replication and temporal distribution of CJD virus in relation to onset of the disease and to determine the important implication of the hematogenous route of virus dissemination

MATERIALS AND METHODS

Mice. Weanling female BALB/c mice obtained from the animal colony of the Frederick Cancer Research Center, Frederick, Md., were used throughout these studies.

Virus. A Japanese strain (Fu) of CJD virus (27), isolated in mice inoculated with human brain from a patient with an atypical case of CJD, at the second mouse intracerebral passage level, was used. The inoculum for mice was prepared from mice with advanced clinical CJD. It consisted of the supernatant of a 10% (wt/vol) suspension of brain in phosphate-buffered saline (PBS), pH 7.4, centrifuged at 2,550 × *g* for 30 min. Thirty microliters (3.1×10^4 mouse 50% lethal doses [LD_{50}]) of this supernatant were inoculated intracerebrally (i.c.) into each weanling mouse.

Removal of infected tissues. Mice were sacrificed at various time intervals as described below. To avoid cross-contamination of tissues, aseptic techniques were employed, and each tissue was processed with a separate set of sterile instruments. Tissues were removed in the following order: urine, blood, brain, spleen, kidney, liver, thymus, and lung. The urine was collected in sterile tubes by pressing the lower abdomen and dialyzed against PBS overnight because of its toxicity. Whole blood was collected by cardiac puncture into heparinized syringes, transferred to a sterile tube, immediately centrifuged at 259 × *g* for 20 min, and then frozen at −70°C overnight (15). The following day, frozen blood was cut with a sterile blade approximately 0.5 cm beyond each side of the buffy coat. Tissues and body fluids were stored at −70°C until used.

Preparation of spleen cell suspension. Groups of 10 mice were sacrificed at 9 and 18 weeks postinfection. The spleens were removed aseptically, minced finely in ice-cold Eagle minimum essential medium (MEM) containing 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20% fetal bovine serum (FBS) (20% FBS-MEM), and filtered through several layers of sterile gauze. Erythrocytes were lysed with 0.83% ammonium chloride. The spleen cells were then washed twice and suspended at a concentration of 10^7 cells per ml in 20% FBS-MEM.

Separation of spleen cells. Spleen cells were separated into macrophages and lymphocytes by the differential adherence of cells to plastic. Five milliliters of the

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spleen cell suspension was incubated in a plastic petri dish (Falcon no. 1007; Falcon Plastics, Oxnard, Calif.) at 37°C for 60 min. Plastic-nonadherent cells (lymphocytes) were removed by pipetting. Plastic-adherent cells (macrophages) were collected by a rubber policeman, washed twice, and suspended in 20% FBS-MEM. The purity of the macrophage fraction was examined by the phagocytic activity. Samples (0.5 ml) of a 5% (vol/vol) opsonized sheep erythrocyte suspension were added to the adherent cells in a plastic petri dish and incubated at 37°C for 120 min. They were then rinsed with PBS, and sheep erythrocyte-ingested cells were determined by staining with Giemsa. More than 95% of cells in this fraction were macrophages. A part of the macrophage fraction was cultured for the study of infectivity. They were maintained in plastic petri dishes (Falcon no. 1007) at 37°C in a humidified 5% $C0_2$ atmosphere, and the medium was changed twice a week. The medium consisted of Dulbecco MEM supplemented with 10% L-cell conditioned medium (19), 10% PBS, and 5% horse serum.

Fractionation of spleen lymphocytes. The plastic nonadherent cells (lymphocytes) were fractionated into T-cell and B-cell fractions by the nylon wool method (16). Briefly, 2×10^8 cells in 2 ml of 20% FBS-MEM were allowed to incubate for 60 min at 37°C in a 20-ml syringe containing 1.5 g of nylon wool. Nylon wool-nonadherent cells were eluted by washing the nylon wool with 60 ml of warm medium; nylon wool-adherent cells were collected by gently squeezing the nylon wool. The efficiency of fractionation was monitored by assaying for T cells by the cytotoxicity test. Briefly, 0.2 ml (2×10^5 cells) of the cell suspension were mixed with 0.2 ml of rabbit anti-mouse thymocyte serum (×256: Microbiological Associates, Rockville, Md.) and 0.4 ml of guinea pig complement (×5; Flow Laboratories, Inc., Rockville, Md.) and incubated at 37°C for 60 min. The percentage of T cells in each cell fraction was more than 95% in the T-cell fraction and less than 20% in the B-cell fraction.

Subfractionation of spleen T cells and B cells. Both T-cell and B-cell fractions were further fractionated into 10 subfractions on continuous density gradients of colloidal silica (Percoll; Pharmacia Fine Chemicals, Piscataway, N.J.). A stock solution of Percoll was first made by mixing undiluted Percoll with 0.15 M NaCl at a ratio of 55:25, and 9 ml of this mixture was centrifuged in a fixed-angle rotor at $20,000 \times g$ for 30 min. After centrifugation, 10^8 cells in 1 ml of 0.15 M NaCl was carefully layered on the top of the gradient and centrifuged at $800 \times g$ for 15 min. Ten 1.0-ml fractions were recovered from the top of gradient and analyzed for density profile and cell count.

Lymphocyte culture. The cells from each lymphocyte fraction and subfraction were cultured in microtiter plates (Falcon no. 3040) for the study of DNA synthesis and the proliferative response to lymphocyte mitogens. Cultures were triplicate 0.2-ml samples of the cell suspension containing 2×10^6 cells per ml in 10% FBS-MEM supplemented with 5×10^5 M 2-mercaptoethanol. Cells were incubated at 37°C for 3 days in a humidified 5% CO_2 atmosphere and pulsed with 0.4 μCi of [^{3}-H]thymidine [6-^{3}H]thymidine, 22 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 24 h before harvesting. For mitogenic stimulation, 20 μl of concanavalin A (ConA, 20 μg/ml; Miles Laboratories, Inc., Elkhart, Ind.), or 20 μl of lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (20 μg/ml; Difco Laboratories, Detroit, Mich.) were added to 0.2 ml of the cell suspension. Acid-insoluble materials from cultured cells were collected on glass fiber filters, and isotope incorporation was measured in a liquid scintillation counter.

In vitro infection of normal spleen cells with virus. Virus for these experiments was prepared as follows. Forty milliliters of a 10% (wt/vol) suspension of infected mouse brain was mixed with 20 ml of Freon 113 (dichloro-difluoromethane; Matheson Gas Instruments, Palo Alto, Calif.) and homogenized in a Sorvall Omni-Mixer (Dupont Instruments, Newtown, Conn.) at the top speed (4,000 rpm) for 10 min. This homogenate was subsequently centrifuged at $50,000 \times g$ for 60 min. The aqueous supernatant (3×10^5 mouse LD_{50} of virus per ml) was used as the source of virus.

The spleen cells were taken from normal BALB/c mice aged 9 weeks and separated into macrophage, T-cell, and B-cell fractions. Part of the T-cell fractions and part of the B-cell fractions were stimulated in vitro with ConA and LPS, respectively. For stimulation of T cells and B cells, 4 μg of ConA or 40 μg of LPS was added to each cell suspension (4×10^6 cells per 2 ml) and cultured for periods ranging from 1 to 3 days. Unstimulated and stimulated spleen cells were adjusted to a concentration of 2.5×10^7 cells per 0.5 ml, and equal volumes of Freon-extracted virus solution were mixed with them. They were then incubated at 37°C for 120 min. After incubation, the cells were washed five times with PBS to remove the unadsorbed virus. As a control, a normal spleen lymphocyte suspension and the virus solution were incubated separately at 37°C for 120 min. They were then mixed and, immediately thereafter, washed five times with PBS.

Assay of infectivity of tissues and cells. Individual tissues from CJD virus-infected mice were homogenized in Tenbroeck grinders to a 10% (wt/vol) suspension in PBS and centrifuged in the cold (4°C) at $2,550 \times g$ for 30 min. Thirty microliters of the supernatants was inoculated i.c. into each weanling recipient mouse. The blood and the urine were inoculated i.c. undiluted. To demonstrate viremia, a single dose of 30 μl of the buffy coat-enriched fraction was inoculated i.c., and 100 μl of other fractions (serum and erythrocytes) collected at weekly intervals were inoculated intraperitoneally. Once every week for 5 weeks, similarly dialyzed urine, collected on a weekly basis from the same mice, was inoculated i.c. at a dose of 30 μl.

Each spleen cell fraction taken from CJD virus-infected mice or cells infected in vitro with CJD virus was adjusted at an appropriate cell concentration in PBS as described below. After treatment of three cycles of freezing and thawing, 30 μl of each cell suspension was inoculated i.c. into each weanling mouse.

After 100 days, mice were examined three times a week for overt signs of the disease, and the examination was continued up to 360 days after inoculation; then asymptomatic survivors were killed for histological study of the CNS. Incubation periods were calculated from the date when unmistakable signs of CJD became apparent. Infectivity was quantitated by the length of mean incubation periods and the incidence of dead mice or by determining the amount of virus in animal tissues or in vitro-infected spleen cells required for 1 mouse LD_{50}.

TABLE 1. Infectivity of tissues from BALB/c mice inoculated i.c. with 3.1×10^4 mouse LD_{50} of CJD virus[a]

Tissue	Infectivity at week after inoculation of virus:						
	1	2	3	6	9	14	18
Brain	174 (5/5)	167 (6/6)	151 (7/7)	133 (5/5)	129 (5/5)	122 (7/7)	112 (7/7)
Spleen	167 (6/6)	133 (6/6)	129 (6/6)	126 (6/6)	126 (6/6)	136 (6/6)	151 (6/6)
Lung			154 (5/6)	139 (6/6)	146 (6/6)	149 (6/6)	186 (6/6)
Liver	—(0/7)	—(0/7)	—(0/7)	—(0/7)	—(0/7)	—(0/7)	271 (5/5)
Kidney	—(0/7)	—(0/7)	—(0/7)	—(0/7)	190 (5/7)	—(0/7)	—(0/7)
Thymus					153 (6/6)	162 (3/3)	181 (5/5)
Blood	—(0/4)	—(0/4)	—(0/2)	281 (5/5)	213 (3/3)	156 (3/3)	142 (5/5)
Urine	—(0/3)	—(0/4)	—(0/3)	—(0/4)	—(0/6)	—(0/6)	—(0/5)

[a] For the infectivity assays, 0.03 ml of the supernatant of a 10% (wt/vol) homogenate of each tissue was inoculated i.c. into weanling mice; to demonstrate viremia, 0.03 ml was inoculated intraperitoneally into mice; 0.03 ml of urine, previously dialyzed against PBS, was inoculated i.c. The results are expressed as the mean incubation days (—, no infectivity) in the recipient mouse, with the number of dead mice/number of mice examined within parentheses. The standard error was >2.5% of the mean incubation period.

RESULTS

Clinical and histological observation of CJD virus-infected mice. Groups of 7 to 10 mice were killed for the study of infectivity of tissues and histology of the CNS at 1, 2, 3, 6, 9, 14, and 18 weeks after inoculation of virus. One group of 10 mice was used for clinical observation. This group of mice exhibited typical signs of the disease such as ruffled fur, weight loss, arched back, plasticity of tail, and bradykinesia at approximately week 16 postinoculation; the mean incubation period in this group was 112 days, with a duration of clinical disease less than 2 weeks.

The characteristic histopathological changes consisting of status spongiosis, astrocytic proliferation and hypertrophy, and neuronal loss were first noted in the CNS at week 9 postinoculation.

Infectivity of tissues from CJD virus-infected mice. Infectivity was detected in the brain and spleen from week 1 after inoculation of virus (Table 1). Of tissues examined, the highest infectivity was present in the brain or the spleen throughout the infection. However, the mode of replication of virus in the spleen was quite different from that in the brain. The infectivity of spleen reached its peak before the appearance of clinical disease and decreased remarkably thereafter; meanwhile, the infectivity of brain increased with time and reached its plateau approximately 18 weeks after inoculation, when all surviving mice were moribund. Notably, the highest infectivity was present in the spleen from week 2 through week 9 postinoculation even when the virus was inoculated i.c. Of other tissues examined, moderately high infectivity was found in the lung from week 3 postinfection and in the thymus from week 9 postinfection (Table 1). Low infectivity was detected transiently in the kidney at week 9 and later in the liver at week 18 post-inoculation. Infectivity was present in the circulating blood from the sixth week and persisted throughout the infection. Infectivity was not detected in the urine.

Infectivity of spleen cells from CJD virus-infected mice. Initially, the infectivity of spleen macrophages, T cells, and B cells was studied at the week 18 after inoculation. In this experiment, a dose of 2×10^6 cells was inoculated i.c. into each recipient mouse. Infectivity was found in each of the cell subpopulations (Table 2). How-

TABLE 2. Infectivity of spleen cell subpopulations taken from BALB/c mice with clinical CJD[a]

Cells	Dose (cells/mouse)[b]	No. of mice dead/total	Mean incubation (days) ± SD
T cell-enriched fraction	2×10^6	7/7	183 ± 2
B cell-enriched fraction	2×10^6	7/7	184 ± 3
Macrophage-enriched fraction	2×10^6	9/9	227 ± 3
Macrophages cultures in vitro for 4 weeks	2×10^6	7/9	276 ± 3
Macrophages cultures in vitro for 8 weeks	2×10^6	0/9	

[a] Each spleen cell subpopulation was prepared from mice 18 weeks after an i.c. inoculation of 3.1×10^4 mouse LD_{50} of virus.

[b] Cells were inoculated i.c. after three cycles of freezing and thawing.

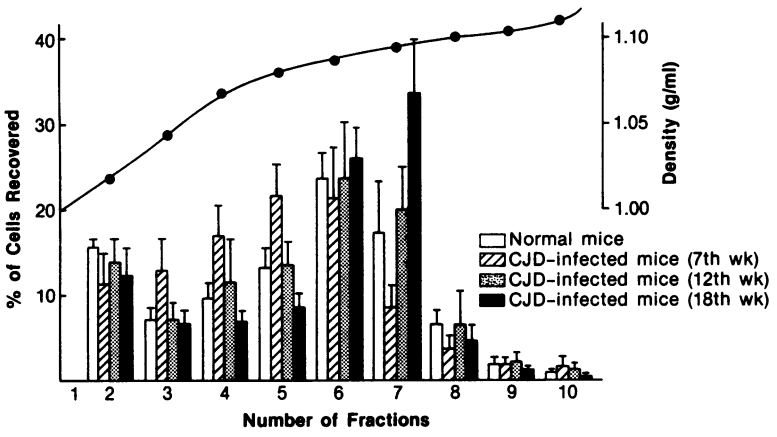

FIG. 1. Density and percentage of spleen cells recovered in each fraction of a continuous density gradient of Percoll. The cells were taken from mice at week 7, 12, and 18 after an i.c. inoculation of 3.1×10^4 mouse LD_{50} of CJD virus and from normal mice at the same age. Ten 1.0-ml cell fractions were recovered and numbered from the top of gradient. The pattern of distribution of cells throughout the gradient was essentially identical among normal mice with different ages.

ever, the mean incubation period for mice inoculated with the macrophage fraction was delayed more than 40 days from those of mice inoculated with lymphocyte fractions. The highest concentration of virus was prsent in lymphocyte fractions. The infectivity of macrophages was also studied after 4 and 8 weeks of in vitro culture. Infectivity was found in the macrophages after 4 weeks of culture, but the mean incubation period for the recipient mouse was prolonged more than 50 days from that of mice inoculated with the uncultured macrophages (Table 2). Infectivity was not detected in the macrophages after 8 weeks of culture.

Infectivity of spleen lymphocyte subpopulations from CJD virus-infected mice. In an attempt to determine which of the spleen lymphocyte subpopulations were most infectious, spleen lymphocytes obtained from CJD virus-infected mice at week 18 postinoculation were separated into 10 fractions on a continuous density gradient of Percoll. The density profile of Percoll gradient and the percentage of lymphocytes recovered in each 1.0-ml fraction are shown in Fig. 1. Viable cells were not found in the superficial fraction (fraction 1), which consisted of 0.15 M NaCl layered on the top of gradient, whereas the viability of the cells was almost 100% in other fractions. This result showed that dead cells did not enter the gradient of Percoll. The pattern of distribution of cells recovered in each fraction of Percoll gradient in CJD virus-infected mice was almost similar with that of normal mice of the same age (Fig. 1).

The infectivity of cells from each cell fraction could only be tested by the i.c. inoculation of 10^5 cells per mouse because of low recoveries of cells in fractions 9 and 10 (Fig. 1). The infectivity was found in all fractions except fraction 9 (Table 3). It is evident that lymphocytes from lower-density fractions (fractions 2 to 5; densities, 1.03 to 1.08 g/ml, respectively) were more infectious than those from higher-density fractions (fractions 6 to 10; densities, 1.09 to 1.12 g/ml, respectively). The highest infectivity was found in lymphocytes from fraction 3 (density, 1.05 g/ml) and fraction 4 (density, 1.07 g/ml) as shown by both the shortest mean incubation periods and the highest incidences of dead mice in the recipient mice (Table 3). Morphologically, large lymphocytes or blastoid cells were observed in lower-density fractions, and small lymphocytes were observed in higher-density fractions. This observation was confirmed by the experiment on the lymphocyte thymidine uptake. The level of [^{3}H]thymidine uptake of lymphocytes from fractions 2 to 5 was higher than that of lymphocytes from fractions 6 to 10 (Table 3). Furthermore, the fractionation of in vitro mitogen-activated spleen T and B cells from normal mice by Percoll showed that 90% of activated lymphocytes were included in fractions 2 to 5; fractions 3 and 4 contained more than 70% of these activated cells (Fig. 2).

Infectivity of spleen T cells and B cells from CJD virus-infected mice. The infectivity of spleen T cells and B cells from CJD virus-infected mice were further analyzed by the density separation with Percoll. This experiment was performed in mice at week 9 after inoculation when the spleen contained the highest infectivity throughout the infection. In these experiments two serial 1.0-ml fractions were mixed and tested for their infectivity because of very

TABLE 3. Infectivity of spleen lymphocyte subpopulations taken from BALB/c mice with clinical CJD[a]

Lymphocyte fraction	Infectivity[b]		[^{3}H]thymidine uptake of cells[c] (cpm)		
	No. of mice dead/total	Mean incubation (days) ± SD	Unstimulated	Stimulated with:	
				ConA	LPS
Unfractionated	5/8	221 ± 9	706	50,072	12,584
Fraction 1					
Fraction 2	7/8	225 ± 25	640	93,716	18,763
Fraction 3	8/8	190 ± 7	2,397	46,960	11,021
Fraction 4	8/8	183 ± 2	5,460	87,692	27,852
Fraction 5	6/8	221 ± 5	1,108	106,126	28,142
Fraction 6	4/8	226 ± 4	507	94,425	14,512
Fraction 7	2/8	242 ± 9	135	105,430	5,942
Fraction 8	1/8	216 ± 0	104	66,740	2,608
Fraction 9	0/8		470	57,988	2,101
Fraction 10	1/7	248 ± 0	199	67,456	2,134

[a] Spleen lymphocytes were prepared from mice 18 weeks after an i.c. inoculation of 3.1×10^4 mouse LD_{50} of virus. Spleen lymphocytes were separated into 10 fractions on a continuous density gradient of Percoll. Fractions were numbered from the top of the gradient.

[b] Lymphocytes were inoculated i.c. into the recipient mouse as a concentration of 10^5 cells per mouse after three cycles of freezing and thawing.

[c] Lymphocytes were harvested after 3 days of culture. For mitogenic stimulation 0.4 μg of ConA or 4 μg of LPS was added to each cell culture (4×10^5 cells per 0.2 ml).

low recoveries of cells in fractions 9 and 10 (Table 4). Infectivity was found in both T-cell and B-cell fractions, but much higher infectivity was found in the B-cell fraction. Evidence of higher infectivity of the B-cell fraction than the T-cell fraction was shown by shorter mean incubation periods, higher incidences of dead mice, and the lesser number of cells required for mouse LD_{50} in the recipient mice (Table 4). The highest infectivity was found in lower-density B-cell fractions (fractions 2 to 4; densities, 1.03 to 1.07 g/ml, respectively). In the case of T cells, cells from lower-density fractions were also more infectious than those from higher-density fractions.

Concurrently, the mitogenic ability of these infected spleen T cells and B cells was studied (Table 4). The background [^{3}H]thymidine uptake was much higher in cells from the B-cell fraction than those from the T-cell fraction. The highest background [^{3}H]thymidine uptake was found in cells from fractions 3 and 4 of the B-cell population, in which the highest infectivity was also detected. In both cell fractions, cells from lower-density fractions incorporated [^{3}H]thymidine more actively than those from higher-density fractions. The proliferative response of spleen T cells and B cells to each mitogen was well preserved. There was no difference in the level of backgorund [^{3}H]thymidine uptake and proliferative response to mitogens between normal spleen cells and CJD virus-infected ones.

In vitro infection of normal spleen cells with the virus. The susceptibility of spleen cells to CJD virus was also studied by means of in vitro coincubation of cells with the virus for 120 min at 37°C. In this experiment, unstimulated macrophages, unstimulated T cells and B cells, and in vitro mitogen-activated T cells and B cells were studied for their susceptibility to the virus. Table 5 shows the difference in the quantity of virus present in each cell fraction. Macrophages and unstimulated T cells and B cells required approximately 10^5 cells for one mouse LD_{50}, whereas with 3-day activated T cells and B cells fewer than 10^3 cells were required for one mouse

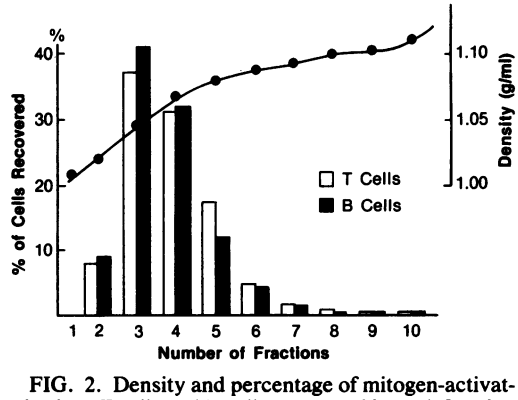

FIG. 2. Density and percentage of mitogen-activated spleen T cells and B cells recovered in each fraction of a continuous density gradient of Percoll. The cells were taken from normal BALB/c mice. For mitogenic stimulation 4 μg of ConA or 40 μg of LPS was added to each T-cell or B-cell culture (4×10^6 cells per 2 ml). After 3 days of culture, cells were fractionated into 10 1.0-ml fractions with Percoll gradient. Fractions were numbered from the top of gradient.

TABLE 4. Infectivity and thymidine uptake of spleen T cells and B cells taken from CJD virus-infected BALB/c mice[a]

Spleen lymphocyte subpopulation	Infectivity of cells[b]			[^{3}H]thymidine uptake of cells[c] (cpm)		
	No. of mice dead/total	Mean incubation (days) ± SD	No. of cells for mouse LD_{50} (log_{10})	Unstimulated	Stimulated with:	
					ConA	LPS
T cells						
Unfractionated	5/6	233 ± 17	4.0	490	87,054	
Fractions 1 and 2	5/7	219 ± 4	4.0	342	54,674	
Fractions 3 and 4	7/7	227 ± 17	4.3	785	99,333	
Fractions 5 and 6	2/6	239 ± 5	5.0	313	74,128	
Fractions 7 and 8	1/7	250 ± 0	5.0	257	59,676	
Fractions 9 and 10	0/7			172	29,013	
B cells						
Unfractionated	16/18	203 ± 15	2.6	1,091		27,270
Fractions 1 and 2	5/7	211 ± 15	2.6	2,181		23,274
Fractions 3 and 4	6/7	181 ± 10	2.6	5,473		63,604
Fractions 5 and 6	6/7	212 ± 17	3.5	1,944		44,878
Fractions 7 and 8	3/6	224 ± 2	5.0	350		16,787
Fractions 9 and 10	4/6	233 ± 13	4.5	362		8,568

[a] Spleen T and B cells were prepared from mice 9 weeks after an i.c. injection of 3.1×10^4 mouse LD_{50} of CJD virus. They were fractionated into 10 fractions on a continuous density gradient of Percoll. Fractions were numbered from the top of gradient.

[b] Mice were inoculated i.c. with 3.3×10^5 cells per mouse.

[c] For mitogenic stimulation, 0.4 μg of ConA or 4.0 μg of LPS was added to each cell culture (4×10^5 cells per 0.2 ml).

LD_{50}. No virus was demonstrated in the cells used as controls.

DISCUSSION

The present studies demonstrate the mode of replication and temporal distribution of virus in CJD in mice. Replication of a Japanese strain (Fu) of CJD virus was indistinguishable from that of scrapie virus; in scrapie in mice the virus is present in the brain, spleen, thymus, and lung at high titers and in the kidney and liver at low titers (6, 22). The present studies also demonstrated the persistence of viremia from the early stage of infection in CJD. This result confirmed the earlier observation of Manuelidis et al. (20) in CJD in guinea pigs. The presence of infectivity in the peripheral blood was also reported by our group (12) and others (3) in scrapie and by Tateishi et al. (27) in CJD. In contrast, several investigators have reported their faillure to detect infectivity in the blood when whole blood (24) or serum (6) were tested for infectivity. The possibility of contamination of infectious tissues into blood can be ruled out in the present studies because the blood was taken by direct cardiac puncture. The successful demonstration of viremia in the present studies was obtained by serial i.c. inoculation of buffy coat, suggesting that in the peripheral blood maximum titer of virus exists in leukocytes (buffy coat), rather than in the serum or erythrocytes. The persistence of viremia from the early stage of infection in the present studies would also indicate that the blood is the most likely vehicle for the secondary dissemination of virus from the initial replication sites in CJD.

The highest infectivity from the weeks 2 through 9 post-inoculation was observed in the spleen even when the virus was inoculated i.c. A number of findings can be construed as supporting this result. In scrapie in mice, in addition to the findings of earliest rise in titer in the spleen irrespective of route of infection (6, 22), there is an increased incubation period in splenectomized or spleenless mutant mice by the intraperitoneal route of inoculation (7, 8), suggesting that there is an initial replicative phase of the infection in the spleen. The question therefore arises as to which spleen cell subpopulations are involved in the initial extraneural replicative phase of the infection. The present studies on the infectivity of spleen cells at the early (week 9) and late (week 18) stages of the infection demonstrated that lower-density lymphocytes contained a high concentration of virus. Further, spleen lymphocytes from lower-density fractions of the Percoll gradient were highly enriched in blastoid cells synthesizing DNA actively. Accordingly, it is assumed that developing lymphocytes, i.e., the blastoid cells, play an important role as the initial replication site of virus in the pathogenesis of spongiform encephalopathies. Experiments of in vitro infection of spleen cells with CJD virus supported this view.

TABLE 5. Infectivity of spleen cell subpopulations infected in vitro with CJD virus[a]

Spleen lymphocyte subpopulations	No. of mice dead/total[b]	Mean incubation (days) ± SD	Amount of cells for mouse LD_{50}[c]
Unstimulated cells			
Macrophages	1/4	238 ± 0	>5.0
Unfractionated lymphocytes	5/6	210 ± 14	4.5
T cells	6/7	207 ± 17	4.4
B cells	1/7	204 ± 0	>5.0
1-day stimulated cells			
T cells	5/6	195 ± 3	3.9
B cells	7/7	180 ± 2	4.0
2-day stimulated cells			
T cells	7/7	168 ± 2	3.6
B cells	7/7	171 ± 5	3.5
3-day stimulated cells			
T cells	7/7	170 ± 7	2.7
B cells	7/7	165 ± 7	2.5

[a] The cells were taken from normal BALB/c mice. For stimulation of T cells and B cells, 4 μg of ConA and 40 μg of LPS, respectively, were added to each cell culture (4×10^6 cells per 2 ml). Both unstimulated and stimulated cells (2.5×10^7 cells per 0.5 ml) were coincubated with CJD virus (1.5×10^5 mouse LD_{50} per 0.5 ml) at 37°C for 120 min. The cells were then washed five times with PBS, adjusted to concentrations of 10^3 to 10^6 cells per 3 ml, and treated with three cycles of freezing and thawing.

[b] The incidence of dead mice and mean incubation period in the recipient mouse inoculated intracerebrally with 10^5 cells per mouse.

[c] The amount of cells needed for one mouse LD_{50} (log titer). The cells were inoculated i.c. at concentrations of 10^5 cells per mouse.

The activation of spleen lymphocytes with mitogens enhanced the susceptibility of cells to the virus. Lavelle et al. (18) reported that in scrapie virus-infected mice the highest infectivity was detected in large spleen cells from lower-density fractions of discontinuous albumin density gradients, and the separation of macrophage-enriched fraction did not result in significant enrichment of scrapie virus-infected cells. Moreover, the susceptibility of mice to scrapie was reduced after steroid administration (23), whereas mitogenic stimulation of the host enhanced susceptibility to the disease (4). The mechanisms of these phenomena are not understood, but possible explanations are that these immunological treatments eliminated or increased activated lymphocytes providing the initial replication site for virus. This view is not surprising since it is well known that activated lymphocytes have the capacity to support replication of diverse types of conventional viruses (5, 17, 25, 29).

As for the difference in the infectivity between T cells and B cells in the present studies, one possible explanation for this difference could be that genetically there is a higher susceptibility of B cells to CJD virus. However, the experiments of in vitro infection of spleen cells with the virus showed that there was no difference in susceptibility between T cells and B cells. The most probable explanation for this difference is that lower-density B cell fractions were highly enriched in blastoid cells having the capacity to support virus replication.

There exists a possibility that the contamination of free virus or dead cells introduced artifacts in the infectivity titer of each cell fraction; however, the densities associated with the highest infectivity, i.e., 1.05 to 1.07 g/ml, were absolutely different for the findings of buoyant density studies of cell-free scrapie virus showing peak infectivity at densities of 1.14 to 1.30 g/ml (2, 26). Concerning the possible contamination of dead cells into each cell fraction, we demonstrated that dead cells did not enter the gradient of Percoll. Gmelig-Meyling and Waldman (14) also reported no entrance of dead cells into Percoll gradients. Therefore, we assume that the difference in the titer of infectivity resulted from the difference in specific infectivity of each cell fraction.

Recently, Garfin et al. (10, 11) reported that the proliferative response of spleen lymphocytes to LPS was significantly suppressed in scrapie virus-infected BALB/c and C3H/HeJ mice. Their findings suggest that this unconventional virus has a capacity to exert a biological influence on host cells. To test this hypothesis, the responsiveness of CJD virus-infected spleen lymphocytes to mitogens was analyzed in detail. Spleen lymphocytes were fractionated into T cells and B cells and thereafter further fraction-

ated into five fractions by continuous density gradients of Percoll. The results of these experiments showed that CJD virus did not exert any biological influence on the ability of T cells and B cells to respond to mitogens. Our previous experiments of coincubation of normal spleen cells with CJD virus in the presence of ConA or LPS for periods ranging from 2 to 5 days also showed that the virus exerted no influence on the mitogen-driven lymphocyte proliferative response.

The strain of CJD virus used in the present studies is different from other strains in that this strain was associated with considerable status spongiosis of the white matter of the patient's brain as well as the brains of experimentally infected animals, and it could be transmitted into small rodents (27, 28). However, fundamental physical and chemical properties and biological behaviors are essentially the same with other strains of CJD virus and scrapie virus (27, 28). Therefore, the general phenomenon in the pathogenesis of CJD in mice may be identical to that in primates. Accordingly, of particular significance to our understanding of CJD infection in humans is the finding of the wide dissemination and persistence of high concentrations of virus in extraneural tissues, including blood, long before the onset of clinical disease.

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