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Characterization of IM Virus, Which Is Frequently Isolated from Cerebrospinal Fluid of Patients with Multiple Sclerosis and Other Chronic Diseases of the Central Nervous System

JOSEPH L. MELNICK,* SHU-SHENG WANG,† EDWARD SEIDEL, GUILLERMO MUCHINIK,‡ LI-BI ZHANG,§ AND ROBERT E. LANFORD

Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030

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A transmissible agent, the IM virus, antigenically related to the Japanese subacute myelo-optico-neuropathy virus, has been isolated from several human cerebrospinal fluids obtained from American patients with multiple sclerosis and other chronic diseases of the central nervous system. The isolates were propagated in human diploid fibroblast (MRC5) cells, and virus was released into the culture medium in the absence of overt cytolysis. Infection of MRC5 cells resulted in a subtle alteration in the normal growth pattern of the cells. In unstained cultures, the cell changes were so mild that it was necessary to carry out all virus assays under code to eliminate bias. Cells in late passages were more susceptible than vigorously growing cells in early passages. Analysis of the kinetics of replication revealed that newly synthesized progeny virus was first detected about 12 h postinfection, that maximal virus release occurred by 48 h postinfection, and that virus production was persistent throughout an 8-day period. Several inhibitors of DNA synthesis were effective in blocking viral replication, including cytosine arabinoside, iododeoxyuridine, and phosphonoacetic acid. A substantial decrease in infectivity was observed upon treatment of IM virus with ether, suggesting that a lipid-containing structure is essential for infectivity. Ultrafiltration studies approximated the size (diameter) of IM virus to be between 100 and 200 nm.

Recently, we reported the isolation of a virus from the cerebrospinal fluid (CSF) of three Texan patients with multiple sclerosis (MS) and one patient with amyotrophic lateral sclerosis (8). Since then, we have recovered a number of additional American isolates, chiefly from patients with MS but also from patients with other chronic central nervous system diseases. The isolates proved to be antigenically related to a virus recovered in Japan from the CSF of patients with subacute myelo-optico-neuropathy (SMON) (5, 6, 9). The virus has also been reported previously from an American who died of a disease presenting the characteristics of SMON (1). However, until its clinical spectrum is more fully understood, Inoue et al. (5) have suggested that the virus be called IM virus (IMV).

We present here our findings on the characteristics of the new viral isolates. The isolates have been difficult to work with because of the mildness of the changes produced in the host cells in culture. To establish reproducible and reliable viral titrations, we found it necessary to carry them out under code, tedious as this might be. Since all the isolates showed antigenic relationships but not identity (5), we selected one (Baylor isolate no. 9) for detailed investigation. However, many of the tests described below for isolate no. 9 were also run with other isolates and the results were similar. Therefore, only the results with isolate no. 9 are presented.

* Corresponding author

† Present address: Guang Xi Chuong Autonomous Region Anti-Epidemic and Hygienic Station, Nanning, Guang Xi, People's Republic of China.

‡ Present address: Instituto de Investigaciones Hematologicas "Mariano R. Castex," Academia Nacional de Medicina de Buenos Aires, 1425 Buenos Aires, Republic of Argentina.

§ Present address: Institute of Virology, Chinese Academy of Medical Sciences, Beijing, People's Republic of China.

MATERIALS AND METHODS

Cell culture. MRC5 human diploid fibroblasts were obtained from Flow Laboratories, Inc., (McLean, Va.) and cultured as previously described (8). Cell cultures between passages number 30 and 38 yielded the best results in viral titrations. Cells were cultured at 37°C in Eagle basal medium with Earle salts (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO), 0.07% sodium bicarbonate, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Virus culture. The virus isolates studied in this report were obtained from the CSF of patients with MS (8). Stocks of the virus isolates were prepared by infecting MRC5 cells cultured in 75-cm^2 plastic flasks (Corning Glass Works, Corning, N.Y.) with a multiplicity of infection of ca. 0.01 50% tissue culture infective doses (TCD_{50}) per cell. Infected cultures were maintained in Eagle minimal essential medium (MEM; GIBCO) supplemented with 2% inactivated FBS, 0.1% sodium bicarbonate, and antibiotics. Cell-free culture fluids were collected 6 days postinfection and were clarified at 3,000 × *g* for 10 min. The TCD_{50} was calculated by the method of Reed and Muench (10).

To achieve consistency, it was essential to eliminate observer bias. This was done as follows. The experiment was set up, and the virus was distributed in test tubes in conventional fashion ready for inoculation of the cell cultures. Then the tubes were randomly coded by another person, and the code was placed in a sealed envelope. After the scoring of cellular changes in the coded cultures by at least two observers was completed on days 5 and 6, their readings were handed to a third person who broke the code and recorded the results in the presence of the persons who had scored the cultures.

Kinetics of replication. MRC5 cells growing in 25-cm^2 flasks were inoculated with 10^5 TCD_{50} of isolate no. 9 per

culture. The virus was allowed to adsorb for 1 h at 37°C. All cultures were washed three times with maintenance medium and were reincubated in 5 ml of maintenance medium. At the times indicated below, culture fluids were collected and assayed for infectivity.

Ether treatment. Virus was diluted 10-fold in MEM without serum, and ether was added to a final concentration of 20%. The virus and ether mixture, plus an untreated control virus sample, were shaken for 10 min and then were incubated at 4°C for 18 h. The ether phase was removed by evaporation in a petri dish, and the treated and untreated samples were titrated. As controls, poliovirus type 1 and herpes simplex virus type 2 were entered into the study and treated as above.

Sensitivity to inhibitors of DNA synthesis. MRC5 cell cultures growing in 25-cm^2 flasks were inoculated with 10^5 TCD_{50} of isolate no. 9. Virus was allowed to adsorb for 1 h at 37°C, monolayers were washed three times with medium, and cultures were reincubated in 5 ml of maintenance medium containing the appropriate inhibitors. Inhibitors were used at the following concentrations (micrograms per milliliter): cytosine arabinoside, 20; iododeoxyuridine, 30; and phosphonoacetic acid, 100. Culture fluids were harvested 4 days after infection and were titrated for infectivity. Poliovirus type 1 and herpes simplex virus type 2 again served as controls.

Ultrafiltration. Ultrafiltration experiments were performed with Sybron Nalge (pore diameter, 450 and 200 nm) and Millipore (pore diameter, 100 and 50 nm) nitrocellulose membrane filters. The filters were sterilized with UV light. All filters were pretreated with MEM containing 2% FBS to reduce nonspecific adsorption of the virus. Virus isolate no. 9 was diluted 10-fold in MEM containing 2% FBS. A sample for titration was taken before passage through the filters. Samples of the diluted virus were passed through a 450-, 200-, and 100-, or 50-nm filter and titrated for infectivity. In addition to poliovirus and herpes simplex virus, SMON virus (6) served as a control.

Differential centrifugation. Ultracentrifugations were performed in a Beckman L5-50 ultracentrifuge with a type 75 Ti rotor. Isolate no. 9 was diluted 10-fold with MEM containing 2% FBS. Samples were subjected to centrifugation at a speed and time sufficient to generate the desired centrifugal force. The relative centrifugal force (RCF) was calculated by the formula $RCF = r\omega^2/980$, and the approximate sedimentation value (S) of the pellet was determined by the equation $S = k/t$ (4). The supernatants were removed carefully and saved for titration. The pellets were resuspended in 1 ml of MEM containing 2% FBS by vortexing and sonication and were saved for titration. Titration of isolate no. 9 was performed as described above. Poliovirus and simian papovavirus simian virus 40 served as controls.

RESULTS

Virus-induced cellular changes. Infection of MRC5 cells with virus isolate no. 9, originally obtained from the CSF of a patient with MS, resulted in an alteration in the normal growth pattern and morphological appearance of a small proportion of the cells in a culture. The virus-induced cellular changes were most easily observed in roller tubes, at the edges of the cell monolayer. The optimum time to examine cells for virus-induced changes was empirically determined to be 5 to 6 days after infection. The normal parallel orientation of the cells was disrupted in areas, resulting in considerable crisscrossing of the cells. Clear spaces appeared in the monolayer due to the loss of close cellular contact, and some cells became swollen and granulated. Figures 1 and 2 show stained preparations of uninfected and infected cell cultures at low and high magnifications. The virus-induced changes were subtle; coded titrations were always done, and the results were dependent upon close comparisons to mock-infected cell controls.

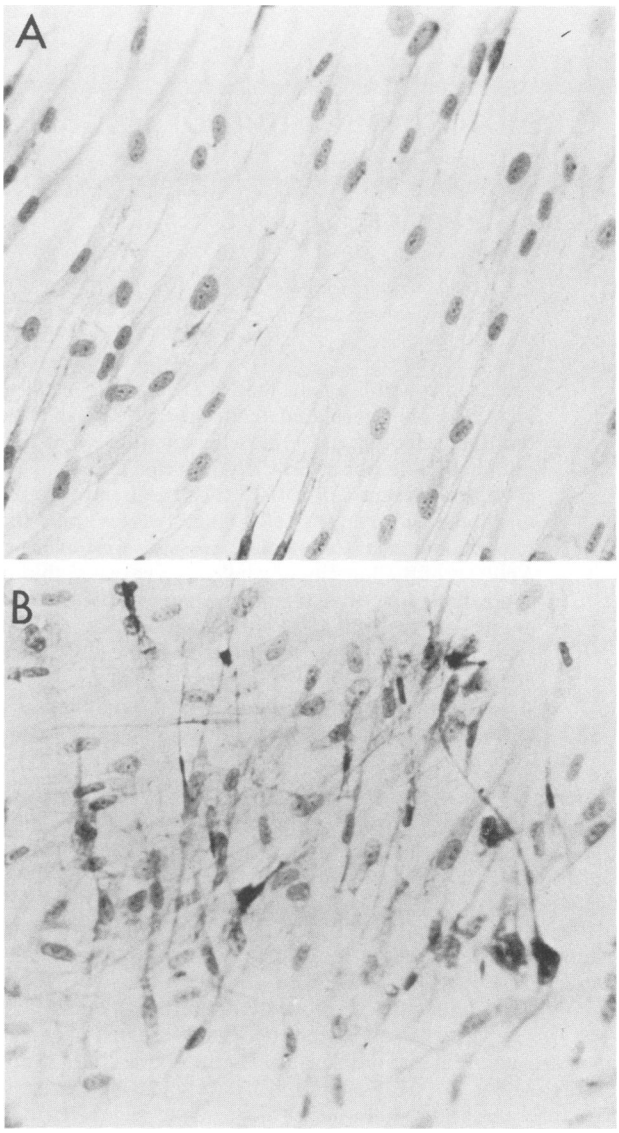

FIG. 1. Alterations of growth in cells infected by IMV isolate no. 9. MRC5 cell cultures growing in 25-cm^2 flasks were inoculated with 10^5 TCD_{50} of virus. After adsorption, cultures were reincubated in 5 ml of maintenance medium. Photographs were taken of stained preparations, 6 days after infection. The parallel growth pattern of uninfected MRC5 cells (A) contrasts with the crisscrossed growth pattern of infected cultures (B).

Influence of cell passage level. As we gained experience with the virus-cell system, we observed that the cytopathic changes were less obvious in vigorously growing cells, which in our hands characterizes the MRC5 line and other human diploid fibroblasts until about passage 30. The results of a test comparing slow-growing MRC5 cells at passage 35 with ES human diploid cells at the P19 level is shown in Table 1. The virus stock yielded a titer of $10^{5.5}$ TCD_{50} per 0.1

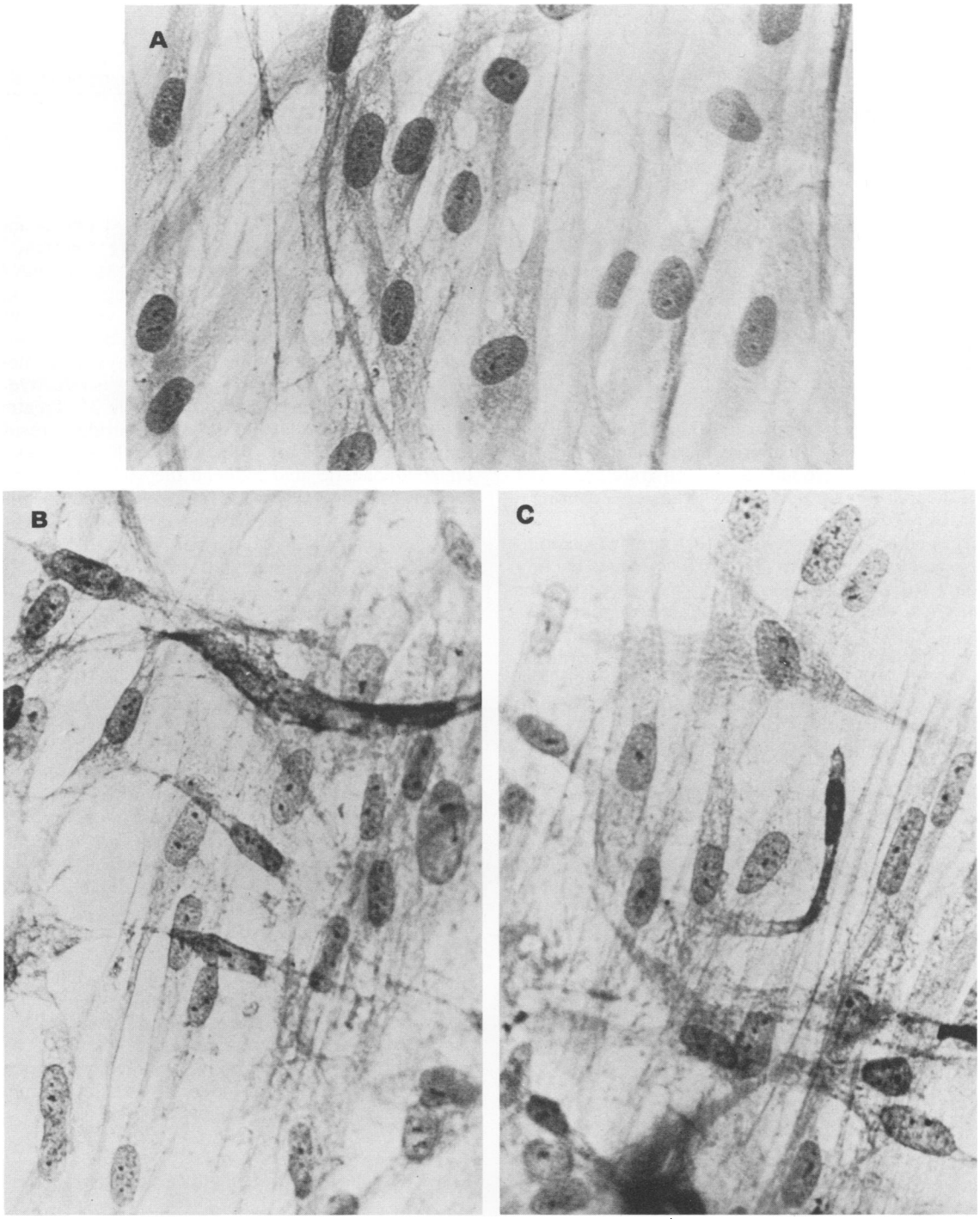

FIG. 2. Stained preparations of MRC5 cells, high magnification. (A) Uninfected cells; (B and C), infected cells.

ml in MRC5 P34 cells but $<10^{3.0}$ TCD_{50} per 0.1 ml in ES P19 cells. Other tests with MRC5 cells in earlier passages also yielded low values, intermediate between those shown for the two titrations in Table 1. The advantages of using the late-passage MRC5 cells are clearly evident.

Kinetics of replication. The experiment was designed to determine the kinetics of appearance of newly synthesized viral progeny. MRC5 cell cultures were inoculated with virus as described above. After infection, the culture fluids were collected at 4, 8, 12, and 16 h and on days 1 through 9. The sample from each time point was assayed for infectivity. A slight increase in virus titer was observed at 12 h with a marked increase apparent by 24 h (Fig. 3). The titer of released virus increased another 100 times by 48 h, when the supernatant fluids contained 10^6 TCD_{50} of virus per ml. In summary, the first infectious progeny were released into the

TABLE 1. Susceptibility of slow-growing MRC5 cells contrasted with that of vigorously growing ES human diploid fibroblasts

Concn of virus stock (per 0.1 ml)	Host cell	Cytopathic effect[a]	
		Day 5	Day 6
10^{-3}	MRC5 P34	±, ±	+, +
10^{-4}		±, +	+, +
10^{-5}		−, ±	−, +
10^{-6}		−, −	−, +
10^{-3}	ES P19	−, −	±, ±
10^{-4}		−, −	−, ±
10^{-5}		−, −	−, −
10^{-6}		−, −	−, ±

[a] Two tube cultures were used at each dilution. The readings on days 5 and 6 were made independently of each other. MRC5 cells were in passage 34; ES cells were in passage 19.

medium by 12 h, maximum virus synthesis was obtained by 48 h, and virus production was persistent through day 8. In other experiments, we were able to demonstrate persistent infection of the cultures for several cell transfers covering a period of many weeks.

Sensitivity to ether. The observation that virus was continuously released into the culture medium was suggestive of a virus budding from the cell surface. Virus was tested for ether sensitivity as described above. A decrease in infectivity of 3 $\log_{10}$ units was observed after ether treatment in comparison to an untreated control (Table 2). As controls, poliovirus proved to be resistant and herpes simplex virus to be susceptible, well-known properties of these agents. The data in this table indicate that an ether-sensitive structure, perhaps a lipid-containing envelope, is necessary for infectivity.

Effect of DNA synthesis inhibitors on replication. The effects of several inhibitors of DNA synthesis were tested in an attempt to determine the type of nucleic acid required for viral replication. Cytosine arabinoside and iododeoxyuridine

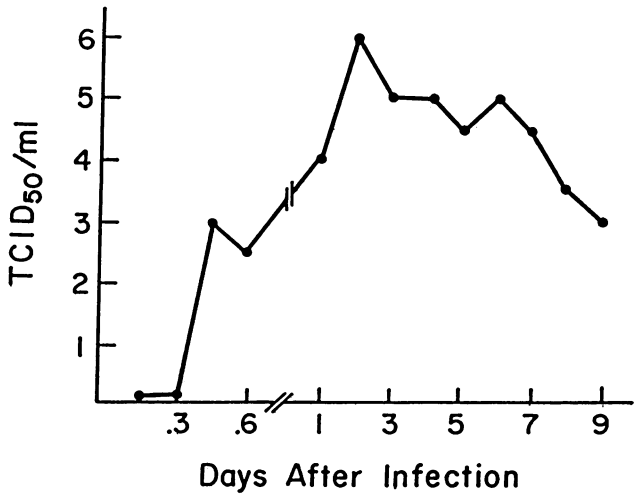

FIG. 3. Kinetics of virus replication. MRC5 cells growing in 25-cm^2 flasks were inoculated with 10^5 TCD_{50} of virus. The cultures were washed three times after adsorption of the virus and were reincubated in 5 ml of maintenance medium. At the indicated times, culture fluids were collected and assayed for infectivity.

TABLE 2. Sensitivity to ether

Virus	$\log_{10}$ TCD_{50} per 0.1 ml		
	Before treatment	After ether treatment	Difference
Isolate no. 9	4.0	1.0	3.0
Poliovirus type 1	6.75	>6.50	None
Herpes simplex virus type 2	7.0	<0.5	6.5

are potent inhibitors of cellular DNA synthesis and of viral DNA synthesis of DNA-containing viruses, whereas phosphonoacetic acid specifically inhibits viral DNA synthesis but only for some DNA-containing viruses (2, 3, 11). MRC5 cells were inoculated with the virus and were incubated in the presence of inhibitors immediately after infection. Culture fluids were collected 4 days after infection and titrated for infectivity. Each inhibitor was effective in reducing the yield of infectious virus (Table 3). Treatment with cytosine arabinoside and iododeoxyuridine resulted in a reduction in titer of 100- and 1,000-fold, respectively. Phosphonoacetic acid reduced the yield of infectious virus further, to a level below the limits of detection. Inhibition of replication by cytosine arabinoside and iododeoxyuridine indicated that either cellular or viral DNA synthesis was required for virus replication; however, the effect of phosphonoacetic acid on the yield of infectious virus suggests that the virus is a DNA-containing virus whose DNA polymerase is sensitive to phosphonoacetic acid. Herpes simplex virus type 2 served as a control for a virus with a DNA genome, and poliovirus type 1 was the control for an RNA-containing virus, since the former is known to be inhibited by DNA inhibitors and poliovirus, with its RNA genome, is known to be resistant to them.

Estimation of size. The approximate diameter of the infectious virus particle was estimated by a series of ultrafiltration experiments as described above. The titer of the virus suspension was not diminished by passage through the 450-nm or the 200-nm filter (Table 4). Passage of the virus suspension through the 100-nm or the 50-nm filter resulted in a marked or total reduction in the amount of virus in the filtrate. These results indicate that the diameter of the virus particle is less than 200 nm and greater than 100 nm. The results are similar to those we obtained in comparative experiments with SMON virus, which was kindly supplied by Y. K. Inoue (Institute for Virus Research, Kyoto University, Kyoto, Japan). Comparative studies with other viruses simultaneously performed indicated the new virus to be medium-sized, larger than poliovirus type 1, and slightly smaller than herpes simplex virus type 2.

TABLE 3. Sensitivity of viral replication to inhibitors of DNA synthesis

Inhibitor[a]	$\log_{10}$ TCD_{50} per 0.1 ml		
	Isolate no. 9	Polio virus type 1	Herpes simplex virus type 2
None	4.0	6.5	6.0
Cytosine arabinoside	2.2	>6.5	<0.8
Iododeoxyuridine	1.0	>6.5	4.0
Phosphonoacetic acid	<0.5	>6.5	<0.8

[a] Inhibitors were added to the medium immediately after adsorption of the virus at the following concentrations (micrograms per milliliter); cytosine arabinoside, 20; iododeoxyuridine, 30; phosphonoacetic acid, 100. Cultures were harvested for poliovirus after 1 day, for herpes simplex virus after 2 days, and for isolate no. 9 after 4 days.

TABLE 4. Ultrafiltration through graded membranes

Filter pore size (diameter, nm)	Titers of filtrates (log_{10} TCD_{50} per 0.1 ml)					
	IMV isolate no. 9[a]			SMON virus	Poliovirus type 1	Herpes simplex virus type 2
	A	B	C			
Control	2.5	3.3	4.0	5.5	6.5	5.6
450	2.5	3.0	3.5	4.2		4.5
200	2.4	2.6	3.5	4.8	7.0	4.5
100	<0.5	1.7	<0.5	1.0	5.0	<0.5
50	<0.5	<0.5		<0.5	4.5	<0.5

[a] A, B, and C represent separate experiments.

Estimation of sedimentation value. The approximate sedimentation coefficient was determined by differential centrifugation as described above. Virus was completely pelleted by centrifugations with force sufficient to pellet particles with values of ≥50S and ≥158S (Table 5). Centrifugation with a force great enough to pellet a particle with a value of ≥50000S did not sediment sufficient virus to be detected by titration. Thus, the virus is estimated to have a sedimentation coefficient between 158S and 500S. Centrifugations of poliovirus (with an established sedimentation value of 158S) and simian virus 40 (with an established value of 240S) under identical conditions were used to confirm the accuracy of our procedure and calculations.

DISCUSSION

The IM virus characterized in this report was recovered from the CSF of a patient with MS. It was selected as typical of the isolations that we have made over the past 4 years, largely from MS patients but also from patients with a variety of central nervous system complaints of a persistent nature. The viruses, isolated from American patients, showed antigenic relationships to the viruses recovered from the CSF of Japanese SMON patients by Inoue et al. (5, 6). The difficulty we encountered before we could develop a reproducible assay for the detection of virus-induced alterations in cellular growth would account for the failure to isolate this virus in some previous investigations. It is essential that all virus titrations be performed under code because of the subtle nature of the virus-induced cellular alterations, particularly in unstained cell cultures. The information obtained about the isolates supports the view that the virus is a distinctive human agent, and further investigation is warranted.

Over a period of 4 years, a large number of coded experiments with different isolates were conducted to determine the properties of the IM virus. The results presented here are those obtained with Baylor isolate no. 9 as representative of the group. Although the results of single experiments are shown, replicate studies with this or other isolates were made by different persons over a period of 4 years with similar results.

The properties that we have found are consistent with those reported earlier by Nishibe et al. (9). The agent is a medium-sized virus, contains essential lipid, and requires DNA for its replication. The estimated size (about 100 nm in diameter) and sedimentation coefficient (over 158S but less than 500S) of the virus suggest that it is in the middle of the viral range. The ether sensitivity of virus infectivity and the presence of virus in the culture medium in the absence of cytolysis suggest that the virus may acquire an envelope by budding through the plasma membrane. Replicating DNA is necessary for viral replication; however, our many attempts at electron microscopic examination of the infected cells or of virus concentrated by ultracentrifugation have failed to reveal recognizable virus particles. Furthermore, numerous attempts to identify the virus by radiolabeling have been unsuccessful. We would be tempted to think of a scrapie-like membranous agent (7), except for the much larger size of our virus.

As of this writing, three antigenic types of the virus have been recognized (5). To avoid any connotation that there is evidence of an etiological relationship of this virus to any specific disease in the American patients, the agent is being temporarily designated as the IM virus (5), with the term SMON reserved only for the strains which had been isolated from the Japanese patients with SMON (6).

TABLE 5. Sedimentation value by differential centrifugation

Centrifugal force (g/h × 10^3)	Sedimentation value of material in pellet	Fraction[a]	Titer[b]
36	≥500	Pellet	<1.0
		Supernatant	5.6
112	≥158	**Pellet**	4.0
		Supernatant	<1.0
362	≥50	**Pellet**	6.0
		Supernatant	<1.0

[a] For each centrifugation run, the location of virus in supernatant or pellet is indicated in boldface.

[b] Log_{10} TCD_{50} per 0.1 ml.

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