INFECTION AND IMMUNITY, Jan. 1979, p. 133-139
0019-9567/79/01-0133/07$02.00/0

Vol. 23, No. 1

Viral Etiology of Age-Dependent Polioencephalomyelitis in C58 Mice

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Received for publication 23 October 1978

The etiology of immune polioencephalomyelitis (IPE) and the mechanisms of resistance to IPE induction were investigated in C58 mice. IPE was found to be induced by a lipid-solvent-sensitive, filterable replicating agent present in line I_b leukemic cell suspensions. IPE was serially transmitted in immunosuppressed mice with filtered extracts of spleens from diseased animals. The IPE-inducing activity of I_b cell extracts was abolished by chloroform or deoxycholate. Gel filtration of I_b cell extracts showed that the IPE agent has a molecular weight of at least 10^7. Electron microscopy of the active fractions from columns and of spinal cord extracts from mice with IPE revealed a virus-like particle, 40 nm in diameter, which is probably the IPE agent. Administration of cyclophosphamide at various times after challenge increased the incidence of IPE in mice, suggesting that IPE is not autoimmune mediated. Immunosuppression resulted in maintenance of high levels of IPE agent in the central nervous system tissue, while immunization resulted in low levels. Moreover, immunized mice produced neutralizing antibodies. These data suggest that antibodies help restrict the amount of IPE agent in the nervous tissue, and that this restriction is required for resistance to IPE induction in C58 mice.

During studies of the immune response of C58 mice to syngenic line I_b leukemia (I_b cells), Murphy et al. (10) found that the injection of inactivated I_b cells induces a paralytic central nervous system disease in old (9 months or older) C58 mice. Histopathologically, the disease is characterized by a mononuclear cell infiltration in the gray matter of the spinal cord and brain stem (5, 6). The disease was thought to result from an autoimmune response of old C58 mice to an antigen common to central nervous system tissue and I_b cells (10, 12). Therefore, it was designated immune polioencephalomyelitis (IPE). The results of early studies indicated that the disease is not induced by latent neurotropic virus or by C-type virus derived from either I_b cells, spontaneous C58 leukemia, or normal C58 tissues (10). Further studies showed that the incidence of IPE can be increased in old and (normally resistant) young C58 mice by immunosuppression (2). The current paper presents evidence that IPE is induced by a filterable replicating agent that is pathogenic for immunosuppressed C58 mice and is distinct from mouse encephalomyelitis (Theiler's) virus. Data are also presented which suggest that IPE is not autoimmune mediated, as originally proposed (10), and that resistance of C58 mice to IPE induction is dependent on immunological restriction of the amount of IPE agent in the central nervous system tissue.

MATERIALS AND METHODS

Mice. C58/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Immunosuppression. Mice from 7 to 10 months of age were given a single intraperitoneal injection of cyclophosphamide (Cytoxan, Meade-Johnson & Co., Evansville, Ind.) at 200 mg/kg, unless otherwise indicated. The average weight of each group of mice was determined, then the concentration of cyclophosphamide (freshly dissolved in water) was adjusted with phosphate-buffered saline (pH 7.2) to deliver the appropriate dose in 1 ml.

Tissue suspensions. Mice with IPE symptoms were exsanguinated, and separate suspensions of the following tissues were made in 4 ml of Hanks balanced salt solution plus 10% glycerol: blood, brain, spinal cord, spleen, mesenteric lymph nodes, peritoneal cells, left liver lobe, left kidney, and both lungs. All tissue suspensions, except spleen, were irradiated to avoid transfer of viable lymphoid cells which can interfere with disease induction (1). Suspensions were exposed to 10,000 R in a model 109 CO^{60} irradiator (J. L. Shepherd & Associates, Glendale, Calif.).

Filtered spleen cell extracts. Spleen cell suspen-

sions from mice with IPE (10^7 cells/ml) or mice moribund from transplanted line I_b leukemia (10^8 I_b cells/ml) were frozen (−70°C) and thawed (37°C) four times, then centrifuged at 500 × *g* for 10 min. The supernatant fluids were filtered through 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.).

Gel filtration. A 0.25-ml portion of the filtered I_b cell extract was chromatographed at 4°C on a column (1.5 by 28 cm), containing Bio-Gel A-15m (Bio-Rad Laboratories, Richmond, Calif.) which has an operation range of 0.3×10^6 to 15×10^6 molecular weight. The column was eluted with phosphate-buffered saline (pH 7.2), and 1-ml fractions were collected. The absorbance of each fraction was determined at 260 and 280 nm.

CsCl density gradient. Five immunosuppressed mice were injected intraperitoneally with a spinal cord suspension from a mouse with IPE, and five others were injected with a spinal cord suspension from a normal mouse. Both groups of mice were sacrificed 12 days after treatment. The spinal cords from each group were suspended in 10 ml of Hanks balanced salt solution by using a homogenizer. The suspensions were frozen and thawed four times, then centrifuged at 10,000 × *g* for 60 min to remove cell debris. Eight milliliters of the supernatants were placed on 30-ml CsCl density gradients (average density, 1.26 g/ml) containing phosphate-buffered saline (pH 7.2) and centrifuged at 25,000 rpm (SW27 Beckman rotor) for 18 h. Fractions were collected through the bottom of the tubes. The refractive index and optical density (254 nm) were determined for each fraction. The refractive index was used to determine the buoyant density (grams per milliliters) of the fractions.

Assay for IPE-inducing activity. Samples were serially diluted 10-fold. One milliliter of each dilution was injected intraperitoneally into each of four mice given cyclophosphamide 24 h previously. Mice were observed for 35 days. Only mice with unequivocal signs of paralysis were scored positive. The titer of the material was defined as the highest dilution causing IPE in at least two of the four mice.

Electron microscopy. Column samples were concentrated by centrifugation at 60,000 rpm in a Beckman SW60 rotor for 3 h before negative staining. A drop of column or gradient fraction was applied to a carbon–Formvar-coated 300-mesh copper grid for 1 min and negatively stained with 2% aqueous phosphotungstic acid (pH 6.3) for 1 min. The air-dried grid was examined in a Philips 300 transmission electron microscope at 80 kV.

Lipid solvent sensitivity tests. The filtered I_b cell extract (10^8 infectious units per ml) was exposed to chloroform (3) or sodium deoxycholate (13) before injection into 10 immunosuppressed C58 mice.

Assay for immunity to IPE. Six-month-old mice were injected intraperitoneally with 10^6 γ-irradiated (10,000 R) I_b cells or with 0.1 ml of GD VII strain mouse encephalomyelitis virus antigen (Microbiological Associates, Bethesda, Md.). One month after immunization, the mice were injected with cyclophosphamide and challenged 24 h later with 10^6 γ-irradiated I_b cells. Resistance to IPE induction with cyclophosphamide and irradiated I_b cells was the indicator of immunity (2).

Assays for antibodies to IPE agent and Theiler's virus. Mice were injected with mouse encephalomyelitis virus antigen or with irradiated I_b cells, as described above. Blood was collected 25 days after immunization. Coded sera were tested for hemagglutination inhibition antibodies to encephalomyelitis virus (11) by Microbiological Associates. Sera were tested for neutralizing antibodies to IPE virus as follows: sera were diluted 1:6, mixed with an equal volume of filtered spleen extract (2×10^3 infectious doses per ml), incubated for 2 h at 37°C, then injected (0.1 ml) into 10 immunosuppressed C58 mice. Sera were considered positive if the incidence of IPE, 21 days after challenge, was reduced by at least 50%.

RESULTS

Transmissibility of IPE agent. A previous study showed that IPE can be induced in old mice with tissues from mice that have developed IPE after an injection of killed line I_b leukemic cells (10). The IPE-inducing activity of the tissues was attributed to carry-over of autoantibody (10) and, later, to carry-over of line I_b cell antigen(s) that cross-reacts with central nervous system tissue (9, 12). An experiment was performed to determine whether IPE is induced, instead, by an infectious agent. Serial 10-fold dilutions of a filtered extract of I_b cells (10^8 cells/ml) were injected into immunosuppressed mice. The mice developed IPE with as little as a 10^{-8}-ml dose; i.e., the equivalent of the extract of one I_b cell. A filtered spleen extract was then made from a mouse which had contracted IPE with this minimum dose. This extract was active in immunosuppressed mice with as few as 10 spleen cells. The minimum-dose transfer procedure was repeated for an additional five passages with filtered extract doses ranging from 1 to 1,000 spleen cells. At each passage, separate undiluted suspensions (0.1 ml) of blood, brain, spinal cord, lymph nodes, peritoneal cells, liver, kidney, and lung from the minimum-dose recipient were injected into immunosuppressed mice. All of these tissues induced IPE. Tissues from uninfected immunosuppressed mice did not induce the disease. Thus, it is apparent that IPE is induced by a filterable replicating agent.

Gel filtration of IPE agent. The results of previous studies (9, 12) suggested that the IPE agent had a molecular weight between 64,000 and 165,000. Such a small molecular weight is incompatible with the thesis that IPE is induced by an infectious agent. Thus, an experiment was performed to obtain a rough estimate of the molecular size of the IPE agent. The I_b cell extract was fractionated on a Bio-Gel A-15m column that had an operational range of 0.3×10^6 to 15×10^6 molecular weight. The fraction obtained in the void volume contained virtually all of the IPE-inducing activity, indicating that

the IPE agent has a molecular weight of at least 10^7 (Fig. 1).

Electron microscopy of active column and gradient fractions. Electron microscopic examination of the active column fraction revealed a virus-like particle, 40 nm in diameter, as well as the expected C-type particles and cellular debris. Since our transfer studies demonstrated the presence of the IPE agent in the spinal cords of mice with IPE, spinal cord extracts, made with Hanks balanced salt solution, were clarified for electron microscopy by density centrifugation. The bulk of the material placed on the gradient remained on the top. The material penetrating into the gradient (buoyant density, 1.21 to 1.30) was pooled, tested for IPE-inducing activity, and concentrated by diluting with phosphate-buffered saline and centrifuging at 25,000 rpm (SW27 rotor) for 18 h. The pooled fraction obtained from infected mice had IPE-inducing activity and contained the 40-nm virus-like particle (Fig. 2), whereas the pooled fraction from uninfected mice did not have IPE-inducing activity or the 40-nm particle. These data suggest that the 40-nm particle is the IPE agent. Figure 2 illustrates negatively stained particles obtained from spinal cords that were extracted with Hanks balanced salt solution containing 0.25% sodium deoxycholate. The size and morphology of the particles is similar to that of papova (lipid-solvent resistant, DNA) virus and toga (lipid-solvent sensitive, RNA) virus groups. The particles have a dense core, ca. 24 nm in diameter, and a rigid outer shell. This latter structure appears to be breaking up in a significant number of particles. This lability might be caused by sodium deoxycholate sensitivity of the particles (see below) or the physical forces applied during the preparation of the specimen.

Lipid solvent sensitivity of the IPE agent. Theiler's virus (8), which is commonly found in mouse colonies, differs considerably in pathogenicity from the IPE agent. Moreover, it is smaller than the 40-nm particle that we suspect of being the etiological agent of IPE. However, it does produce lesions in the central nervous system similar to that of IPE. Thus, lipid-solvent sensitivity tests and cross-immunization studies (below) were done to determine whether Theiler's virus is involved, directly or indirectly, with the induction of IPE. To determine whether the IPE agent, like Theiler's virus, is resistant to lipid solvents, aliquots of the filtered I_b cell extract were exposed to chloroform or sodium deoxycholate. Both of these lipid solvents completely abolished the IPE-inducing activity of the extract (10^8 infectious units per ml), indicating that the IPE agent contains lipids

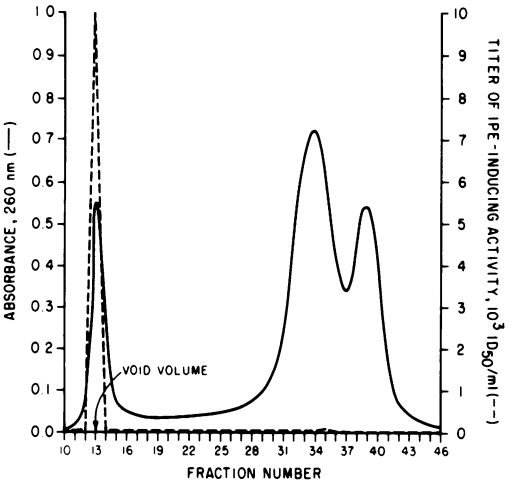

FIG. 1. *Gel filtration of line I_b leukemic cell extract on a Bio-Gel A-15m column. A 0.25-ml aliquot was layered on a column (1.5 by 28 cm) and eluted with phosphate-buffered saline (pH 7.2) at 4°C. The absorbance (——) of each 1-ml fraction was determined at 260 nm. Each fraction that had a detectable absorbance was titrated for IPE-inducing activity (- - -) in immunosuppressed C58 mice.*

that are essential for its functional integrity. Therefore, Theiler's virus or any other lipid-solvent-resistant virus, could not be the direct cause of IPE.

Lack of antigenic cross-reactivity between IPE agent and mouse encephalomyelitis (Theiler's) virus. To investigate the possibility that IPE was induced by a mixed infection including Theiler's virus, cross-immunization studies were done, and the C58 mouse stock was tested for natural Theiler's virus infection by sampling at random for antibodies to Theiler's virus.

First, a method for detecting antibodies to the IPE agent was developed. An experiment was performed to determine whether sera from immunized C58 mice would neutralize the IPE agent and thereby protect immunosuppressed C58 mice. Groups of 3-month-old mice were immunized with a single injection of IPE agent (10^6 irradiated I_b cells) or hyperimmunized with two injections, 1 month apart. Sera were collected and pooled 25 days after the last injection. The IPE agent (100 infectious doses) was incubated with saline or sera from normal, immunized, and hyperimmunized mice, then injected into cyclophosphamide-treated mice, as described above. Figure 3, representative of two experiments, shows that only the sera from immunized and hyperimmunized mice were protective.

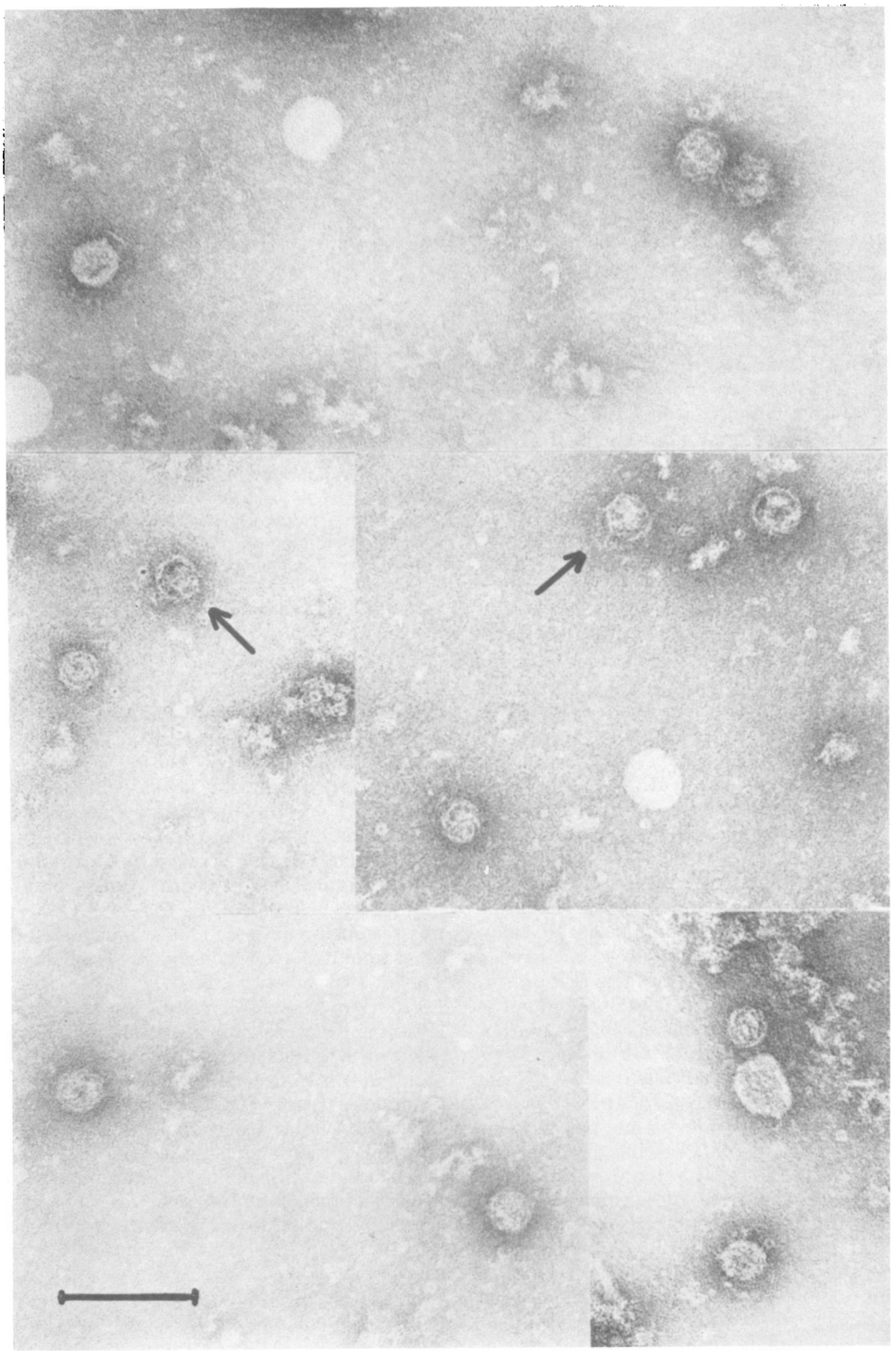

FIG. 2. *CsCl density gradient-clarified spinal cord extracts that were negatively stained with 2% phosphotungstic acid (pH 6.3). Typical 40-nm virus-like particles are shown. The arrows indicate particles in which the outer shell appears to be in the process of disintegrating. Bar, 100 nm.*

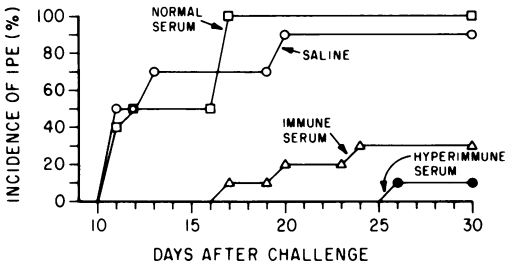

FIG. 3. *Neutralization of IPE agent with immune sera. A preparation of IPE agent (100 infectious units) was incubated with saline* (○), *normal serum* (□), *immune serum* (△), *and hyperimmune serum* (●) *and then injected into groups of 10 immunosuppressed C58 mice. The incidence of paralysis was observed for 30 days.*

Sera from mice, injected with Theiler's virus or IPE agent, were tested for antibodies to each of these viruses 25 days after immunization. Table 1 shows that Theiler's virus and IPE agent failed to induce antibodies against each other. Moreover, mice injected with Theiler's virus were not protected against IPE induction. Thus, by these criteria the two agents have no antigenic cross-reactivity. There was also no detectable antibody to Theiler's virus (evidence of infection) in the C58 mouse stock. These data indicate that Theiler's virus is not involved in the induction of IPE.

Failure to suppress the induction of IPE by immunosuppression. The optimum time of cyclophosphamide treatment for suppressing virus-induced immune-mediated central nervous system diseases is after viral challenge (4, 7). Since IPE was suspected of being immune mediated (9, 10, 12) and virus induced, attempts were made to suppress the induction of IPE with cyclophosphamide given at various times after challenge with IPE agent (10^6 irradiated I_b cells). Groups of 3-month-old (normally resistant) and 14-month-old (normally susceptible) C58 mice were given a single injection of cyclophosphamide (200 mg/kg) on different days, ranging from 1 day before to 7 days after challenge. In both sets of mice, immunosuppression at the various times increased the incidence of IPE instead of decreasing it (Table 2). Thus, a more rigorous attempt was made to suppress IPE induction. Groups of 6-month-old (normally resistant) C58 mice were given 200 mg of cyclophosphamide per kg 1 day before challenge, then 250 mg/kg at different times (4 to 8 days) after challenge. The double-injection regimen uniformly increased the incidence of IPE, regardless of administration time (Table 3). These data strongly suggest that IPE is not autoimmune mediated.

Opposite effects of immunization and immunosuppression on the amount of IPE agent in the central nervous system. In a previous study (2), it was shown that young C58 mice can be made susceptible to IPE induction by immunosuppression. However, prior immunization of mice makes them resistant to IPE induction, even when immunosuppression is used. Thus, an experiment was performed to determine whether prior immunization and/or immunosuppression of C58 mice would have any effect on the amount of IPE agent detectable in the central nervous system tissue after challenge. Young (6-month-old) mice were immunized with IPE agent (10^6 irradiated I_b cells), or were not injected. One month later, half of the mice in each group were given cyclophosphamide. Twenty-four hours later, all four groups of mice were challenged with IPE agent (10^6 irradiated I_b cells). The brain and spinal cord from two mice in each group were harvested for IPE

TABLE 1. *Test for antigenic cross-reactivity between Theiler's virus and IPE agent*

Immunization material[a]	Proportion of mice with antibodies to:	
	Theiler's virus[b]	IPE agent[c]
IPE agent (irradiated I_b cells)	0/16	10/10
Saline	0/11	0/10
Theiler's virus (mouse brain)	6/10	0/10
Normal mouse brain	0/10	ND[d]

[a] Six-month-old C58 mice were immunized with the indicated material 25 days before collection of blood.
[b] Hemagglutination inhibition titer, ≥20.
[c] Neutralization titer, ≥6.
[d] ND, Not done.

TABLE 2. *Effects of one injection of cyclophosphamide on resistance to IPE induction*

Day of cyclophosphamide injection[a]	Incidence of IPE in:	
	3-month-old C58 mice	14-month-old C58 mice
−1	10/10	14/14
0	9/10	11/11
1	9/10	13/13
2	10/10	12/12
3	7/10	12/12
4	9/10	10/11
5	10/10	13/13
6	6/10	11/12
7	5/10	14/14
—[b]	0/10	10/13

[a] Cyclophosphamide was given before or after the injection of IPE agent (10^6 irradiated I_b cells) on day 0.
[b] —, Cyclophosphamide not given.

TABLE 3. *Effects of two injections of cyclophosphamide on resistance to IPE induction*

Cyclophosphamide[a]		Incidence of IPE	
200 mg/kg on day −1	250 mg/kg on day:	Proportion	Mean day ± SD
+	−	15/15	12.1 ± 1.6
+	4	15/15	12.6 ± 1.5
+	5	15/15	11.7 ± 1.3
+	6	15/15	13.5 ± 3.2
+	7	15/15	12.5 ± 2.1
+	8	15/15	14.0 ± 4.0
−	−	0/15	NA[b]

[a] Groups of 6-month-old C58 mice were given cyclophosphamide 1 day before challenge with IPE agent (10^6 irradiated I_b cells) and at different times after challenge. +, Cyclophosphamide given; −, cyclophosphamide not given.

[b] Not applicable.

assay at various times (4 to 25 days) after challenge. The amount of IPE agent in the central nervous system of non-immunized mice decreased from day 4 to day 11 after challenge and remained lowered thereafter (Fig. 4). Immunized mice had a reduced amount of IPE agent throughout the entire observation period. Non-immunized mice given cyclophosphamide maintained the initial high level of IPE agent. Immunized mice given cyclophosphamide had a reduced amount of IPE agent throughout the observation period, possibly because of already present antibody and/or the resistance of the secondary immune response to immunosuppression. In general, immunosuppression resulted in maintenance of high levels of IPE agent in the central nervous system, while immunization resulted in low levels, suggesting that the resistance of C58 mice to IPE induction is dependent on immunological restriction of the amount of agent in the nervous tissue.

DISCUSSION

The data presented establish that IPE is caused by a filterable replicating agent contaminating line I_b leukemic cell suspensions. The poor inducibility of IPE led previous investigators to the conclusion that IPE is not induced by a virus (10, 12). For example, IPE cannot be induced by intraperitoneal challenge of normal young C58 mice or in normal young or old mice of the BALB, C3H/He, C57BL/6, and AKR strains. Moreover, young C58 and BALB mice are even resistant to an intracranial injection of brain suspensions obtained from old C58 mice with IPE. However, the results of subsequent studies (1, 2) suggested to us that the etiological agent of IPE might be a virus. For example, it was found that young and old C58 mice can be made susceptible to IPE induction with irradiated I_b cells by immunosuppression with X-irradiation or drugs (2). This indicated that IPE might be caused by a virus which is pathogenic for C58 mice that are immunosuppressed by the normal aging process or by treatment with immunosuppressive agents. Accordingly, cyclophosphamide-treated C58 mice were used in the present study to investigate the possible viral etiology of IPE. The serial transmissibility of IPE with filtered spleen extracts showed that IPE is induced by a filterable, replicating agent. Gel filtration studies showed that the IPE agent has a molecular weight of at least 10^7. Electron microscopic examination of preparations that had IPE-inducing activity revealed a 40-nm virus-like particle, which is probably the IPE agent. The results of lipid-solvent sensitivity tests and cross-immunization studies indicated that mouse encephalomyelitis (Theiler's) virus is not involved in the induction of IPE.

To explain the cause of neuron destruction in IPE, previous investigators postulated that the lesions are a result of breakage of tolerance to central nervous tissue antigens by a cross-reactive antigen(s) in I_b cells (9, 10, 12). This explanation seemed appropriate (6) because of the

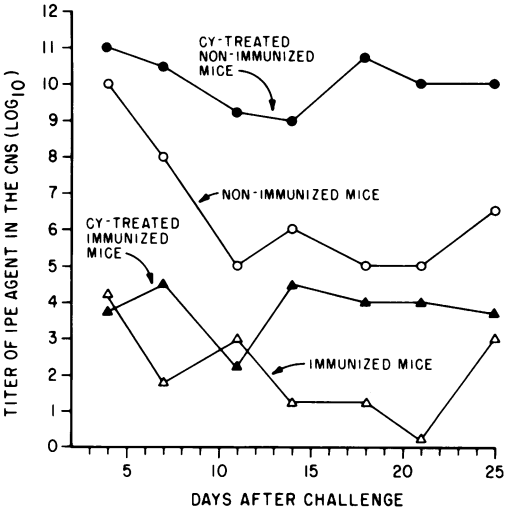

FIG. 4. *Effects of immunization and/or immunosuppression on the amount of IPE agent in the central nervous system of C58 mice. Groups of 7-month-old mice, immunized with IPE agent (10^6 irradiated I_b cells) or nonimmunized, were injected with cyclophosphamide (CY) or saline 24 h before challenge with IPE agent. The central nervous system tissue of two mice from each group (each suspended in 4 ml of Hanks balanced salt solution) was titrated for IPE agent at the indicated times after challenge. The geometric mean of the titers (ID_{50}/ml) is given for CY-treated nonimmunized mice (●), nonimmunized mice (○), CY-treated immunized mice (▲), and immunized mice (△).*

failure to demonstrate viral involvement in IPE induction. Although we have demonstrated that IPE induction can take place in the absence of I_b cells, it is still possible that the IPE agent, which is probably a virus, initiates an autoimmune process. However, most of the evidence does not support an autoimmune (lymphocyte-mediated) mechanism. For example, administration of immunosuppressive therapy, in a manner that would suppress the development of virus-induced immune-mediated central nervous system diseases in mice such as lymphocytic choriomeningitis (4) and the "late" white matter disease induced by Theiler's virus (7), increases the incidence of IPE in susceptible mice. In this regard, it is noteworthy that the "early" disease induced by Theiler's virus results from a cytolytic infection and, like IPE, involves the gray matter and is exacerbated by immunosuppression (7). Perhaps the IPE agent, also, causes a cytolytic infection of neurons and possibly other cells in the gray matter. Alternatively, the destruction of neurons might be the result of a nonspecific inflammatory reaction directed toward the infectious process in the nervous tissue.

The immunological elements which are responsible for resistance of C58 mice to IPE induction have been partially characterized. The resistance to IPE induction is thymus dependent, viz., neonatally thymectomized young adult mice are susceptible to IPE induction (1). The question remains as to which thymus-dependent function(s) is required for protection, e.g., suppressor- or helper-cell function. Since C58 mice are able to make neutralizing antibodies against IPE virus (Fig. 3), it is possible that resistance is dependent on helper T-cell function, which is diminished by thymectomy or the natural aging process. It may be that neutralizing antibody helps restrict the amount of IPE virus in the central nervous system tissue (Fig. 4) and that this restriction is required for resistance to IPE induction. The knowledge that IPE is induced by an infectious agent should facilitate investigation of both the pathogenetic and protective mechanisms involved in IPE.

ACKNOWLEDGMENTS

We thank Patricia A. Dennison, Carolyn A. Dennis, George P. Lampson, and C. Shirley Lin for their excellent technical assistance.

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