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Brain Lysosomal Glycosidase Activity in Immunosuppressed Mice Infected with Avirulent Semliki Forest Virus

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Mice infected with an avirulent strain of Semliki forest virus show an increase in the activity of some of the brain lysosomal glycosidases. The increase in activity of these enzymes has been correlated with the histological, virological, and serological changes that result from the infection in the presence and absence of immunosuppression. Semliki forest virus alone caused the development of a mild encephalitis with perivascular infiltration, microgliosis, astrocyte hypertrophy, and a focal spongiform encephalopathy, together with an increased activity of brain *N*-acetyl-β-D-glucosaminidase and β-glucuronidase. Antilymphocyte serum given after infection marginally affected the course of the disease. Cyclophosphamide markedly delayed the development of the spongy changes and the increase in enzyme activities, but not the perivascular infiltration. It is suggested that the increased activity of the lysosomal glycosidases studied may be linked both to the development of a successful immune response and to the focal spongiform changes produced by the infection.

Attenuated or avirulent strains of some togaviruses have the ability to cause a mild subacute encephalitis without the development of clinical signs. Histological lesions include perivascular infiltration by mononuclear cells, microglial accumulations, astrocytic hypertrophy, and varying degrees of spongiform vacuolation (26, 28, 29). It has been shown recently that the infection of mice with an avirulent strain of Semliki forest (SF) virus can also produce a similar pathological picture 5 or 6 days after inoculation (Mackenzie, Suckling, & Wilson, manuscript in preparation), together with an increase in some lysosomal glycosidase activities (21). In addition, the spongiform changes may be initiated or become more pronounced in hosts that show immune deficiency, or after immunosuppression (25, 27).

In these previous investigations, the pathological changes provoked by SF virus appear to fall midway between the severe encephalitis caused by lethal infections with encephalitogenic viruses and the astrocytosis and spongiform vacuolation without preliminary encephalitis that is characteristic of the slow virus diseases, such as scrapie. In a similar way, the elevation of some of the brain glycosidase activities produced by avirulent SF virus falls midway between the situation in lethal infections, where no elevation in activity occurs (11, 20), and scrapie, where very large elevations have been recorded (15).

The purpose of the work presented in this paper is to investigate the involvement of the lysosomal glycosidases in the pathogenesis of avirulent SF virus in mice. However, using indirect techniques, it is difficult to interpret the relative contribution made to the rise in enzyme activity either by infiltrating cell types or by the cells of the brain itself. After initial experiments using a relatively nonselective form of immunosuppression, gamma irradiation, we found that the time course of glycosidase elevation was altered. Using more selective treatments, we have now attempted to identify the role of the immune response in eliciting histopathological and glycosidase changes, Antilymphocyte serum (ALS) or cyclophosphamide was used after virus infection, since it is generally accepted that they deplete, respectively, the numbers of sensitized T and B lymphocytes available (10, 14, 22). A preliminary report of this work has appeared previously (20).

MATERIALS AND METHODS

Infection of mice. Female Swiss A_2G mice, 28 days old, were used. SF virus (strain A774) was kindly provided by C. J. Bradish of the Microbiological Research Establishment, Porton, Wiltshire, England. The virus had a titer of $10^{7.5}$ intracerebral mean lethal doses ($ICLD_{50}$)/0.02 ml in suckling mice but was not lethal for mice 25 days of age or older. Before use, dilutions of stock virus were made in 0.75% bovine serum albumen in phosphate-buffered saline (BAPS) at pH 7.3. Groups of mice were inoculated intraperitoneally (i.p.) with $10^{3.9}$ $ICLD_{50}$ of SF

virus in 0.1 ml of BAPS or with 0.1 ml of BAPS alone. Virus infectivity was determined by diluting 10% (vol/vol) dilutions of blood in serial 10-fold steps. Four serial dilutions were used for each titration, and 0.02 ml was inoculated intracerebrally into groups of five 2- to 4-day-old mice. The suckling mouse $ICLD_{50}$ was calculated by the method of Reed and Muench (16).

Immunosuppressive agents. For some preliminary experiments, batches of mice were treated with whole-body gamma irradiation from a ^{60}Co source using a sublethal dose of 500 rads. Cyclophosphamide (Endoxana; WB Pharmaceuticals, Bracknell, Berkshire, England) was given i.p. on postinfective day 1 at a dose of 200 mg/kg. Cyclophosphamide at this dosage produced reduction in spleen weight within 24 h to 20% of its normal value, which lasted 6 days.

ALS was prepared by following the technique of Levey and Medawar (10) in New Zealand white rabbits injected on three occasions with a suspension of mouse thymus tissue. Antiserum produced by this method is capable of delaying skin homograft rejection for at least 10 days (10). The ALS used was tested for specificity in vitro and found to lyse 95% of the cells in a homologous thymus cell suspension but only 30% of the cells of a homologous spleen cell suspension. In addition, the ALS was tested in vivo for potency by its ability to prolong the average survival time of mice infected with a lethal dose of Langat virus. Previous work in our laboratory has established that immunosuppressive procedures capable of affecting T cell-mediated immune responses regularly produce a statistically significant increase in the average survival time of these mice by 1 to 2 days (S. Jagelman, manuscript in preparation). The ALS used in our present experiments increased the average survival time by 1.2 days ($P = 0.005$). The ALS was administered 1 and 5 days after SF virus infection, with a dose of 0.25 ml i.p. on each occasion.

Immune serum production and humoral antibody assay. Immune serum was prepared by inoculating a number of adult mice with SF virus ($10^{4.0}$ $ICLD_{50}$/0.1 ml, i.p.), repeating the dose after 14 days and exsanguinating after a further 7 days. The homologous antiserum produced in this way had a serum neutralization index of 4.5. In the experiments described here, 0.5 ml was given i.p. 4 days after infection.

Humoral antibody was assayed in serum from the experimental mice by hemagglutination inhibition tests using the micromethods of Sever (19). Antigens were prepared by the sucrose-acetone method (7) and by protamine sulfate precipitation (23).

Sampling techniques. Infected and control mice were sampled at appropriate times in groups of five to seven animals. The mice were ether anesthetized and exsanguinated, and a sterile 10% suspension of blood in BAPS was prepared for virus assay; serum was collected for antibody assays. The spleens were removed and weighed; the brains were removed and divided sagittally, and half was placed in 10% formol saline. The other half was homogenized gently in BAPS by 10 strokes with a motor-driven Teflon-in-glass homogenizer to provide a sterile 10% (wt/vol) homogenate of brain for virus and biochemical assays. These samples were kept frozen at −70°C until required.

The brains from a number of mice infected i.p. with SF virus alone were removed 4 days after infection, as described previously, but were divided into cerebellum and cerebral cortex, with the exclusion of the midbrain and brain stem. The cerebellum and cerebral cortex of each of these brains were assayed for virus infectivity as described previously. The results of these assays are shown in Table 2.

Glycosidase assays. Glycosidase activities were measured by their ability to hydrolyze *p*-nitrophenyl glycosidase substrates (15) in the presence of 0.1% Triton X-100. The *p*-nitrophenol derivatives of *N*-acetyl-β-D-glucosamine, *N*-acetyl-β-D-galactosamine, and β-D-glucuronic acid were obtained from Koch-Light Ltd., Colnbrook, Bucks, England. Results were calculated by reference to an optical density curve generated with standard solutions of *p*-nitrophenol.

Histological techniques. Half of each brain was processed by standard procedures in which 5-μm sections were stained with hematoxylin and eosin, and others were stained with a silver impregnation method for observing microglia and oligodendrocytes. Occasionally, 10-μm frozen sections were stained by Cajal's method to allow the visualization of astrocytes.

RESULTS

Effect of immunosuppression on the disease process. Estimations of blood virus titers on the infected mice showed that a viremia persisted 5 days after infection only in those mice treated with cyclophosphamide. On day 14 virus was still detectable in 5/5 mice, on day 18 it was detectable in 1/5 mice, and on day 25 clearance was complete. Figure 1 demonstrates the effect of ALS and cyclophosphamide treatment on the brain virus titers of mice infected with SF virus. ALS caused a slight delay in the reduction of brain virus titer, but cyclophosphamide administration allowed virus to persist for 18 days. After cyclophosphamide treatment there was no increased mortality, but there was an increase to about 50% in the occurrence of hind-limb paralysis.

In Fig. 2 the hemagglutination inhibition titers for SF virus are given for the same groups of mice. Up to day 18 the cyclophosphamide-treated mice possessed significantly less antibody; the ALS-treated mice showed a response not significantly different from that seen in mice infected with virus alone.

Histological appearance after immunosuppression. In mice given SF virus alone, perivascular accumulation of leukocytes, generally sparse, were seen in several brain areas. Focal lesions, including spongiform vacuolation, as-

trocyte hypertrophy, and microgliosis (the accumulation of cerebral macrophages), most frequently affected the white matter of the cerebellum, midbrain, and brain stem. The astrocyte hypertrophy and spongiform vacuolation were the lesions that took longest to disappear. The midbrain lesions may be responsible for the incidence of paralysis recorded. Figure 3 illustrates the distribution of the focal lesions.

Lesions appearing after the administration of ALS did not differ greatly from those appearing after the administration of virus alone, but there was a tendency for perivascular infiltration to be slightly more severe and glial nodules appeared in several sections. Whereas cyclophosphamide delayed the onset of the appearance of focal vacuolation, the vacuolation was never less severe than in those mice given SF virus alone, and in half the samples examined its severity was increased markedly. The mononuclear perivascular infiltration was present at a slightly reduced level. The time of appearance of the key histological and biochemical lesions is indicated in Table 1.

The administration of antiserum parenterally after virus infection did not appear to cause lesions differing in type or severity from those induced by the virus alone.

Glycosidase levels after immunosuppression. The activities of *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase, and β-glucuronidase were measured in brain ho-

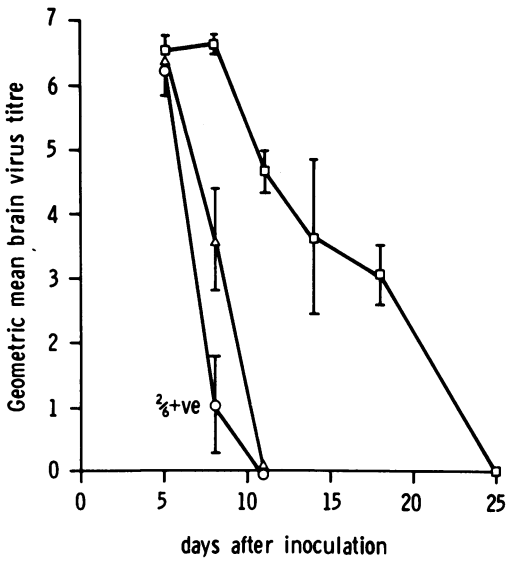

FIG. 1. *Effect of immunosuppression on the brain virus titers of mice infected with avirulent SF virus. Virus titers are expressed as* $\log_{10}$ *suckling mouse IC* LD_{50}. *Symbols: ○, untreated; △, treated with ALS; □, treated with cyclophosphamide.*

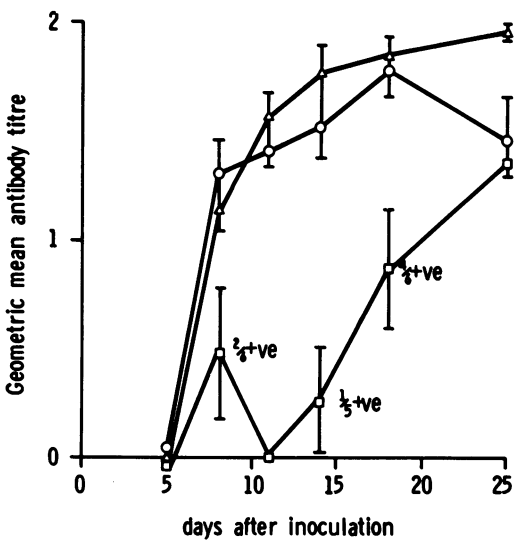

FIG. 2. *Effect of immunosuppression on the blood antibody (hemagglutination inhibition) titers to SF virus. Antibody levels are expressed as* $\log_{10}$ *of the reciprocal of the hemagglutination inhibition titer. Symbols: ○, untreated; △, treated with ALS; □, treated with cyclophosphamide.*

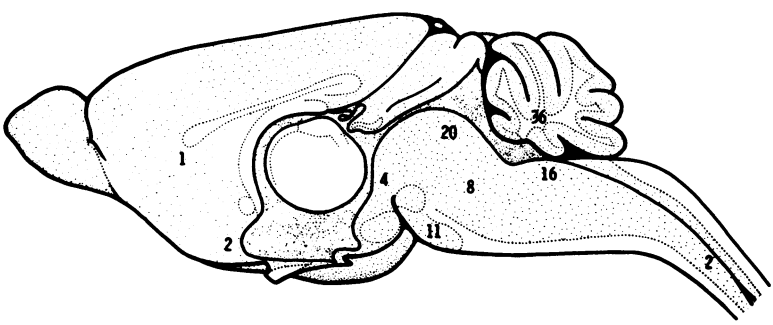

FIG. 3. *Distribution of focal lesions in brain infected with avirulent SF virus. Numbers represent the percent occurrence of lesions appearing in 47 brain sections.*

mogenates from mice infected with SF virus alone and after treatment with ^{60}Co irradiation, ALS, and cyclophosphamide. The homogenates showed essentially similar alterations in activity for each of the three glycosidases measured, so only changes in *N*-acetyl-β-D-glucosaminidase are given in Fig. 4. In addition, only these three glycosidases out of a total of 10 tested (*N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase, β-D-glucuronidase, α-D-glucosidase β-D-glucosidase, α-D-galactosidase, β-D-galactosidase, α-L-fucosidase, β-D-mannosidase, β-D-xylosidase) showed a significant rise in activity after infection with virus alone. The control levels of activity of *N*-acetyl-β-D-glucosaminidase were between 11 and 12 mg of *p*-nitrophenol liberated/h per g (wet weight) of brain tissue. The immunosuppressive treatments in the absence of virus did not significantly affect this control value. In Fig. 4 activities were significantly above the appropriate control between 8 and 25 days after infection with SF virus alone (all points, $P < 0.001$); between 10 and 18 days after infection with SF virus preceded by irradiation (all points, $P < 0.001$); between 8 and 25 days after infection with SF virus followed by ALS (day 8, $P < 0.05$; day 11, $P < 0.005$; day 18, $P < 0.001$; day 25, $P < 0.005$); and with SF virus followed by cyclophosphamide on days 25 ($P < 0.01$) and 35 ($P < 0.01$) after infection. The administration of immune serum parenterally after virus infection had no effect on the pattern of enzyme rise.

TABLE 1. *Time of appearance of lesions after avirulent SF virus infection alone and after immunosuppressive treatments*

Treatment	Days after infection at which significant changes first appear		
	Focal vacuolation	Perivascular infiltration	Increase in enzyme activity
Virus alone	8	5	8
Virus + ALS	8	5	8
Virus + cyclophosphamide	14	5	Some at 14 and 18; all at 25

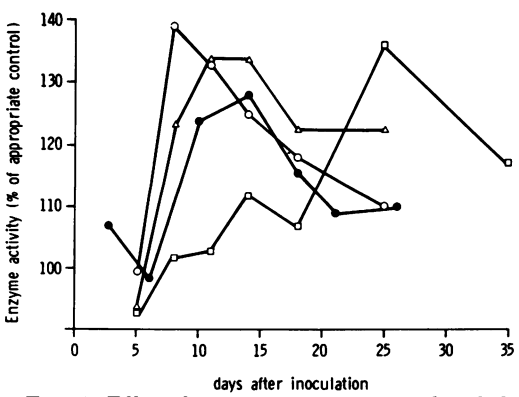

FIG. 4. *Effect of immunosuppression on the whole brain activity of N-acetyl-β-D-glucosaminidase in mice infected with avirulent SF virus. Symbols: ○, untreated; ●, treated with irradiation; △, treated with ALS; □, treated with cyclophosphamide.*

Correlation of biochemical and histological results. The comparison of enzyme levels and pathological changes in separate halves of individual brains may not always provide a close correlation of results, especially where more than one cell type is responsible for contributing to the total activity of that enzyme. However, assessing changes on a group basis, which also tends to minimize the inevitable individual variation in immune responsiveness, we have shown that there was good correlation between the activity of the lysosomal glycosidases and some of the histological changes we have observed. Enzyme activities correlated particularly well with the appearance of focal vacuolation, and with the associated astroytosis and microglial involvement in the early stages of the infection (Table 1). Later in the disease process, when the perivascular infiltrations had disappeared and microglial reaction had subsided, vacuolation, astrocytic hypertrophy, and elevation of the enzyme activities were still apparent.

DISCUSSION

Several studies have reported rises in the glycosidase activity of the brain during various encephalitic conditions (1, 3, 9, 11, 12), but the cause of these increases is often not clear. During an avirulent and inapparent SF virus infection, it has been shown that the activity of *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase, and β-glucuronidase first increases 7 days after infection (21). The time after infection at which the glycosidases increase in activity can be altered by host immunosuppression (20), and we have shown that gamma irradiation appeared to produce a peak of activity later than that caused by SF virus alone (Fig. 4). These results suggested that glycosidase levels were increased as a response to some event that occurred during the immune response. Since gamma irradiation produces a nonspecific immunosuppression, further experiments were performed using more specific immunosuppressive agents.

ALS, active against T lymphocytes (14), did not appear to alter the changes in glycosidase levels or the development of humoral antibody to a significant degree. Cyclophosphamide effectively delayed the appearance of humoral

antibody for several days (Fig. 2), presumably by its suppressing effect on B lymphocytes (22). This would almost certainly account for the delay in clearance of virus from the brain by approximately 14 days (Fig. 1). The increase of glycosidase activity was also delayed, by about 13 days (Fig. 4). The brain glycosidase levels could not be made to rise earlier by the parenteral administration of immune serum to infected mice. Serum given in this way is unlikely to be able to cross the blood brain barrier effectively.

We therefore suggest that one requirement for the initial production of increased glycosidase activity is the presence of competent B lymphocytes within the perivascular cuffs, producing antibody locally, which subsequently neutralizes intracerebral virus antigen. This type of antibody production within the brain substance has been reported previously in togavirus infections (8, 17).

Histologically, avirulent SF virus infection produces at least four types of lesions; perivascular infiltration, microgliosis, astrocyte hypertrophy, and focal spongiform vacuolation (6, 13, 18, 26). All the cell types involved in the lesions represent possible sources of increased lysosomal enzyme production. Leukocytes and macrophages are known to have the ability to produce increased amounts of lysosomal enzymes in vitro when stimulated by immune complexes (24), especially when antigen and antibody are present in equivalent amounts (4). Infiltrative and microglial cells (cerebral macrophages) increase in number greatly early in the infection and contribute to the early enzyme changes both by virtue of their increase in numbers and because of immunologically induced activation.

Later in the disease process, when the generalized inflammatory response had diminished, other cell types probably contributed more to the whole brain glycosidase level. Astrocytic hypertrophy and its associated spongiform vacuolation tended to be found only in the midbrain, brain stem, and cerebellum (Fig. 3). It was also these areas that showed the largest increase in glycosidase activity (21; Mackenzie et al., in preparation). It has been reported that astrocytes are capable of markedly increasing their lysosomal enzyme activities in multiple sclerosis brain (1). In our work the astrocytic and associated spongiform changes were first apparent at 7 days postinfection and continued for 6 weeks. Not only did areas that showed these changes correspond to the areas of most severe biochemical change, but they were also exposed to higher levels of virus earlier in the infection (Table 2). One explanation of these higher levels of antigen is that certain cellular areas were better able to support virus replication, but another is that antibody levels in these areas were lower.

The focal lesions without inflammatory changes are similar to the lesions seen in scrapie-infected mouse brain, which are also associated with high glycosidase levels (11, 15). Scrapie apparently does not elicit a detectable host immune response (2), and in this disease increases in lysosomal enzyme production may be evoked as a functional response to the disruption of the cell membrane structure by the scrapie agent. In our mice, infected with SF virus, a generalized immunological response did occur, but in the particular cerebellar areas of focal astrocyte hypertrophy and spongiform vacuolation we did not observe any inflammatory reaction, perhaps because of a local antibody deficiency, as suggested earlier. It is therefore possible that the raised glycosidase levels that are closely associated with these focal changes are not the direct products of an immunological process but, as in scrapie, may be functional responses to cell membrane, which has been altered by viral antigen.

Finally, neuronal damage may elicit increased enzyme production. Again in scrapie-affected mice increased glucosaminidase activity has been associated with neurones (5), and in our work with SF virus there also appears to be neuronal involvement (S. Pathak, personal communication).

Whereas early glycosidase production is associated with perivascular cuffing and microgliosis during the phase of successful clearance of virus from the brain, the later, persistent changes, associated with astrocyte hypertrophy, spongiform encephalopathy, and some neuronal involvement, may be less than beneficial. In a recent report it has been shown that

TABLE 2. *Brain titers of SF virus in cerebellum and cerebrum 4 days after i.p. inoculation* [a]

Sample no.	Virus titer ($SMICLD_{50}$/0.02 ml)	
	Cerebellum	Cerebrum
1	4.9	5.5
2	7.1	5.7
3	7.5	6.5
4	5.3	5.1
5	6.5	5.0
6	6.4	4.5
7	6.5	6.5
8	6.7	5.7
Mean ± SEM	6.36 ± 0.31	5.56 ± 0.25

[a] Geometric mean titer of cerebellum is significantly higher than that of the cerebrum ($0.01 < P < 0.05$). $SMICLD_{50}$, Suckling mouse $ICLD_{50}$; SEM, standard error of mean.

demyelination can be provoked in the cerebellar white matter by repeated parenteral infections with avirulent SF virus (M. Chew-Lim, A. J. Suckling, and H. E. Webb, Vet. Pathol., in press). The stimulation of increased lysosomal enzyme production during the SF virus infection may play a part in the predisposition of infected brains towards developing demyelination after repeated infections. Work is presently in progress to investigate the link between enzyme production and myelin loss.

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