JOURNAL OF VIROLOGY, Mar. 1986, p. 1139–1144
0022-538X/86/031139-06$02.00/0

Vol. 57, No. 3

Effects of Cyclosporin A on Humoral Immune Response and Resistance against Vesicular Stomatitis Virus in Mice

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Received 29 July 1985/Accepted 26 November 1985

The effect of cyclosporin A (CS-A) on the antiviral humoral response was studied by using vesicular stomatitis virus (VSV); VSV provided the opportunity to simultaneously assess both T-independent and T-dependent antibody responses. The T-independent anti-VSV immunoglobulin M (IgM) response was virtually unaffected, whereas the T-dependent primary anti-VSV IgG response was suppressed by CS-A; in contrast, the secondary IgG response was highly resistant to CS-A. Moreover, once the switch from IgM to IgG had occurred, the primary response also became refractory to suppression by CS-A. We concluded that the effect of CS-A on the primary anti-VSV antibody response was mediated via impairment of a T-dependent mechanism; in contrast, memory T cells or memory B cells or both were quite resistant to the suppressive effects of CS-A. CS-A treatment rendered mice highly susceptible to VSV infection; under CS-A treatment, mortality was 100% after infection via footpads, whereas immunocompetent mice survived. Since CS-A does not impair induction of early T-independent anti-VSV IgM neutralizing antibodies, this high mortality in CS-A treated mice illustrates the crucial role of CS-A-sensitive cells in resistance against VSV.

Cyclosporin A (CS-A) causes remarkable immunosuppression and is widely used in transplantation immunology (6, 7, 27, 31–34). CS-A has also been successfully tested in experimental studies of allergic encephalomyelitis, oxazolone hyperreactivity, autoimmune uveitis and diabetes (5, 6, 16, 18, 20). The selectively inhibitory effect on immune cells at the onset of an immune response obviously might make the recipient of CS-A treatment more susceptible to infections, particularly by viruses. Therefore, studies of antiviral immune responses under CS-A treatment may improve our understanding of mechanisms of CS-A action.

In the present investigations, we have used vesicular stomatitis virus (VSV) to study the effects of CS-A on primary and secondary antibody responses. We observed that the early T-independent immunoglobulin M (IgM) response was insensitive to CS-A; the initiation of T-dependent primary IgG responses was highly sensitive, whereas the already-initiated or the secondary IgG responses were highly resistant to CS-A. CS-A drastically increased the susceptibility of mice to VSV; early paralysis was frequently observed, and VSV infection resulted in a very high mortality.

MATERIALS AND METHODS

Mice. DBA/2 (*H-2*^{d}), C57BL/6 (*H-2*^{b}), and B10.BR (*H-2*^{k}) mice were obtained from the Institut für Zuchthygiene, Zürich, Switzerland; nude (*nu/nu*) and euthymic (+/+) C57BL/6 mice were a gift of the Institut für Medizinische Forschung, Füllinsdorf, Switzerland.

Virus. VSV Indiana (VSV-Ind; Mudd-Summer isolate) and VSV New Jersey (VSV-NJ; Pringle isolate) were provided by D. Kolakofsky, University of Geneva, Geneva, Switzerland. Virus stocks were prepared by infecting confluent BHK-21 monolayers at a low multiplicity of infection. The cultures were fed with minimum essential medium containing 5% fetal calf serum, 1% glutamine, antibiotics (penicillin and streptomycin), and 0.02% $NaHCO_3$. After 20 h of incubation at 37°C in 5% CO_2, the culture supernatants were harvested. The supernatants were clarified by centrifugation at 600 × *g* for 15 min at 4°C and stored in 1-ml portions at −70°C.

CS-A preparation. CS-A was a generous gift from Jean F. Borel, Sandoz Ltd., Basel, Switzerland. The powder was dissolved in absolute alcohol–Tween-80. This solution was suspended in warm phosphate-buffered saline and stored at room temperature, protected from light. Mice were injected daily intraperitoneally (i.p.) with a final concentration of CS-A equalling 60 mg per kg of body weight (15). Control mice were treated with only the similarly prepared solvent.

Immunization protocol and CS-A treatment. VSV-Ind and VSV-NJ were injected intravenously (i.v.) with 10^6 PFU/0.2 ml of inoculum for most of the experiments (unless stated otherwise) to study humoral immune responses. For the injection of VSV into the footpad, an inoculum of 10^7 or 10^5 PFU of VSV-Ind in a 30-μl volume was used.

To explore the effect of CS-A on primary antibody responses to VSV or the clinical cause of infection after VSV inoculation into the footpad, CS-A was injected daily beginning 1 day before infection and continuing for the studied course of the immune response up to day 12. The effect of CS-A on secondary antibody responses was studied by repeated daily i.p. injections from one day before secondary immunization throughout the entire course of the studied secondary immune response. Control mice were injected with solvent alone.

SN test. For the serum neutralization (SN) test, sera from three to five mice were pooled for each sampling time and heat inactivated at 56°C for 30 min, and twofold dilutions were mixed with equal volumes of virus diluted to contain 50 PFU/100 μl. The serum-virus mixture was incubated for 90 min at 37°C under an atmosphere of 5% CO_2 and then transferred onto Vero cell monolayers grown in 24-well

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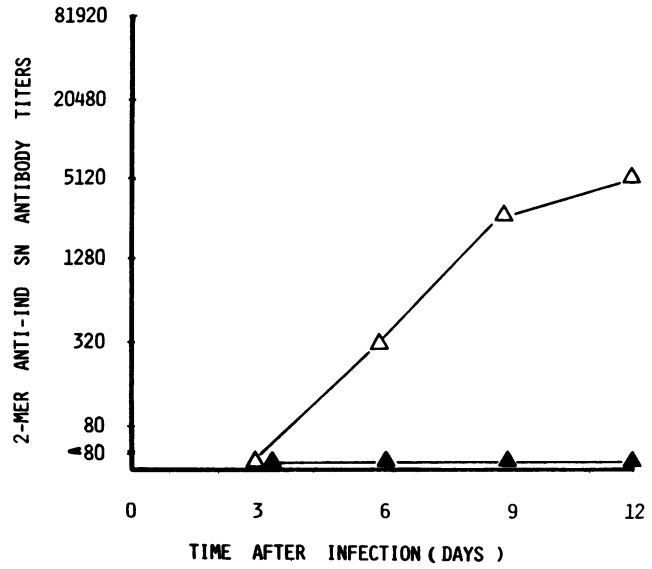

FIG. 1. Effect of CS-A on the primary anti-VSV-Ind IgG antibody response. 2-MER SN antibody responses of CS-A-treated mice (▲) and mice treated with the solvent (△).

plates (no. 3024; Costar, Cambridge, Mass.). After incubation at 37°C for 90 min, the monolayers were overlaid with 2% methylcellulose and 2× Iscove modified Dulbecco medium mixed in equal amounts and then further incubated at 37°C under 5% CO_2 for 48 h in a humid chamber. The overlay was flicked off, and monolayers were stained with 5% crystal violet. Due to the addition of equal volumes of virus in the serum-virus mixtures, the final dilutions of serum were considered one step higher, and the highest dilution of serum which reduced the plaques by 50% was taken as the titer. To determine IgG titers, sera were pretreated with 0.1 M 2-mercaptoethanol (2-ME) in physiological saline for 1 h at room temperature (26). 2-ME-resistant (2-MER) antibody titers were presumed to represent IgG; 2-ME-sensitive (2-MES) antibody titers were taken to represent IgM.

RESULTS

Effect of CS-A on primary antibody response. Daily treatment of mice with CS-A starting 1 day before infection completely abrogated anti-VSV-Ind and anti-VSV-NJ 2-MER SN antibody responses (Fig. 1 and 2). A delayed 2-MER antibody response was detectable when the CS-A treatment was stopped after 8 days; but when the use of CS-A was extended up to 12 days after infection, 2-MER SN antibodies did not appear until at least 3 weeks later. Treatment with CS-A did not impair the early anti-VSV 2-MES SN (i.e., IgM) antibody response when compared with 2-MES SN anti-VSV responses in normal (+/+) mice and nude (*nu/nu*) mice; however, the 2-MES SN response in CS-A treated mice declined more rapidly than that in nude mice (Fig. 3 and 4). We observed that, in contrast to untreated mice, mice that were infected with VSV i.v. and treated with CS-A showed approximately 50% mortality between 2 to 3 weeks after infection, i.e., at the time when anti-VSV IgM antibodies disappeared.

Effect of CS-A on secondary antibody response. In a series of experiments, mice were primed with 10^4, 10^6, or 10^8 PFU of VSV-Ind or VSV-NJ and challenged at different times with the homologous serotypes with or without CS-A treatment (Fig. 5 and 6). Daily treatment with CS-A from day −1 onwards had little effect on the secondary anti-VSV antibody responses with any of the protocols tested. Anti-VSV SN antibody titers peaked at about 80,000 around day 6 after secondary infection regardless of prechallenge titers, dose of priming, or the interval between priming and challenge. The kinetics of secondary anti-VSV antibody responses in groups C and D (long-term memory) developed in parallel in both of the groups treated with CS-A and in control groups (Fig. 5 and 6).

Effect of CS-A on the ongoing antibody response. Although CS-A treatment could totally inhibit the induction of 2-MER

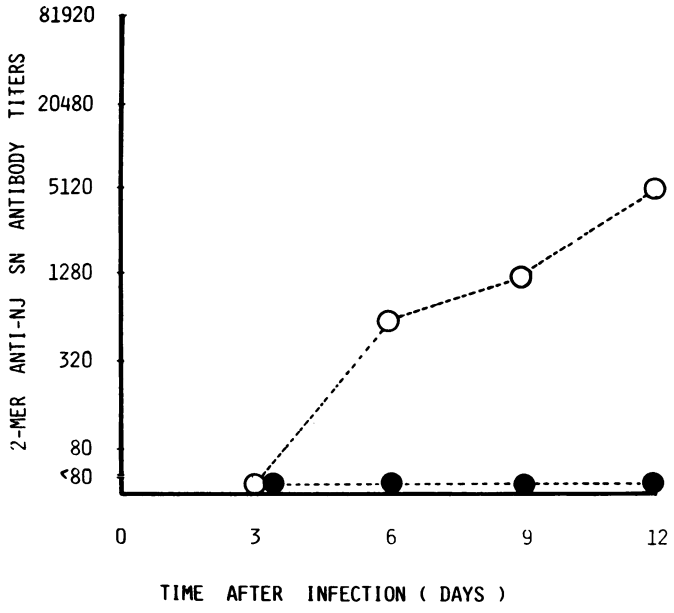

FIG. 2. Effect of CS-A on the primary anti-VSV-NJ IgG antibody response. 2-MER SN antibody responses of CS-A-treated mice (●) and control mice treated with the solvent (○).

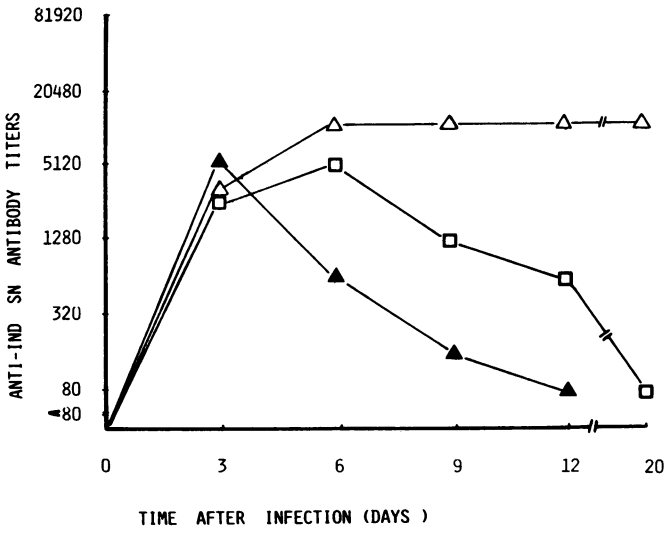

FIG. 3. Comparison of primary anti-VSV-Ind total SN antibody response (without 2-ME treatment) in +/+ mice treated with CS-A (▲), +/+ mice treated with solvent (△), and nu/nu mice (□).

anti-VSV SN antibody responses when used from day −1 onwards, it had no influence on anti-VSV antibody responses when treatment was started on day 7 postimmunization, i.e., after anti-VSV specific 2-MER antibodies had already appeared. Moreover, the CS-A-treated groups and the control groups of mice showed identical kinetics of 2-MER SN antibody responses throughout the period of observation during which CS-A treatment was continued (Fig. 7).

Pathogenesis of VSV infection in CS-A-treated mice. Mice are not natural hosts for VSV. The amount of virus (10^6 PFU) used for immunizing mice i.v. did not cause death; up to 10^8 PFU of VSV inoculated i.v. was not lethal. However, mice primed with 10^6 PFU of VSV i.v. and treated with CS-A from day −1 to day 12 of infection showed approximately 50% mortality 2 to 3 weeks after infection. A few mice which died or showed paralysis of hind legs were subjected to histopathological analysis; besides interstitial pneumonia, we found intracytoplasmic inclusions in some neurons similar to Negri bodies in rabies. The brains of infected CS-A-treated mice which died were found to contain up to 5×10^7 PFU of VSV; no virus was ever found in the brains of i.v. infected untreated mice. VSV infected into the footpad resulted much more frequently in extensive hind leg paralysis and death than did i.v. inoculation. The mean time to death and mortality of mice inoculated with VSV-Ind into the footpad when treated with CS-A daily from 1 day before infection or left untreated are shown in Table 1. DBA/2 mice treated with CS-A showed paralysis on day 3 and died on day 4 or 5, depending on the dose of virus inoculated; untreated DBA/2 (*H-2*^{d}) mice survived without any sign of the disease. Similarly, most C57BL/6 (*H-2*^{b}) or B10.BR (*H-2*^{k}) mice inoculated with 10^7 or 10^5 PFU of VSV and treated with CS-A died between days 8 and 11. Four of six (67%) untreated C57BL/6 mice inoculated with 10^7 PFU developed mild paralysis of hind feet on day 8, but no mortality was recorded up to day 20; by day 14 after initiation of infection, two of the four paralyzed mice had recovered. Untreated C57BL/6 mice inoculated with 10^5 PFU developed no signs of paralysis. One of six untreated B10.BR mice inoculated with 10^7 PFU in the footpad developed paralysis and died on day 15. CS-A treatment had no influence on protection of C57BL/6 (*H-2*^{b}) or of B10.BR (*H-2*^{k}) mice from 10^5 PFU VSV-Ind injected intracerebrally (i.c.); all mice died between days 4 and 5. C57BL/6 (*H-2*^{b}) mice preimmunized with VSV-Ind 5 months earlier or B10.BR (*H-2*^{k}) mice preimmunized with VSV-Ind 3 weeks earlier were not protected against VSV-Ind challenge infections i.c. with 10^5 PFU. These VSV-Ind immune mice all died between days 4 and 6 regardless of whether they were treated with CS-A.

DISCUSSION

CS-A (60 mg/kg) given to mice beginning 1 day before infection with VSV and continuing daily thereafter had the following influence on antibody responses to VSV: no effect on the T-independent early IgM response, suppression of the switch from IgM to IgG, suppression of the primary IgG response, and no effect on secondary IgG responses. Overall, CS-A drastically increased the susceptibility of mice to VSV with respect to both paralysis and mortality.

The immunosuppressive action of CSA has been extensively analyzed over the past 8 years (6, 7, 27, 30–34). It seems to inhibit activation and proliferation of T cells mainly by interfering with the production and release of interleukins (IL) (1, 10, 14, 16, 19, 21), particularly those of IL-2 (16).

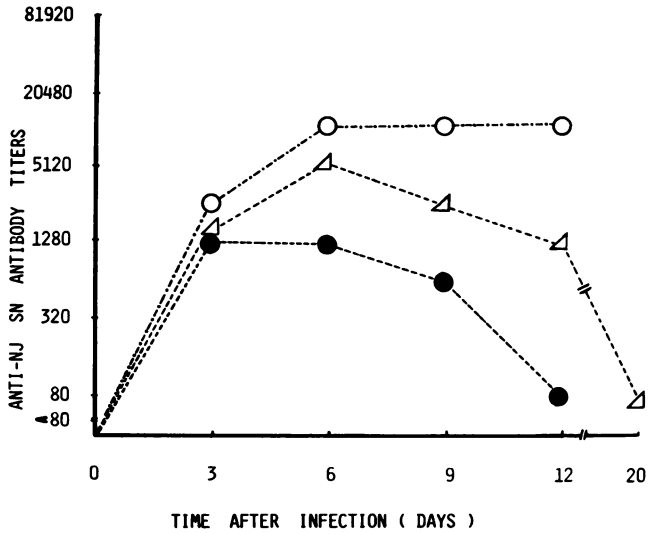

FIG. 4. Comparison of primary anti-VSV-NJ total SN antibody response (without 2-ME treatment) in +/+ mice treated with CS-A (●), +/+ mice treated with solvent (○), and nu/nu mice ( ).

These hormone-like factors regulate T-cell differentiation and proliferation (19). All primary T-cell responses tested so far are drastically reduced or eliminated by CS-A in mice and humans (8, 31). Since primary IgG responses, including the switch from early IgM to later IgG responses, are highly dependent upon helper T cells, the action of CS-A on T cells explains the lack of IgG antibody responses under CS-A treatment. CS-A seems to have no or only marginal direct effects on B cells (17, 31–33).

As has been previously shown in nude mice (11), the initial, very early, IgM response of mice to VSV is T cell independent; this IgM response is resistant to treatment with CS-A (Fig. 3 and 4). VSV has been shown to activate B cells (13); the repetitive arrangement of viral glycoproteins on the viral envelope or on infected cells (24) is probably responsible for this mitogenic action. Our finding that CS-A did not impair the T-cell-independent IgM response is compatible with results from in vitro studies which demonstrated that B-cell proliferation induced by Epstein-Barr virus and bacterial lipopolysaccharides (28) was not impaired. In contrast, other B-cell responses that more or less depend upon help from T cells have repeatedly been found to be reduced by CS-A (4, 6, 17, 28). Our results showing that CS-A suppressed anti-VSV IgG responses confirm these earlier findings.

Previous studies with herpes simplex virus in vivo have shown that antibody responses are refractory to the effects of CS-A (3). Since IgM and IgG responses were not separated on day 6 after infection, that study did not address the possibility that the unimpaired IgM (17) response may have been responsible for the finding. Also, antibody responses to influenza virus have been shown to be temporarily suppressed by CS-A treatment (2, 25); however, it is difficult to evaluate these results, since kinetic studies and titrations were performed in only a few cases.

Secondary anti-VSV IgG responses were resistant to CS-A. This appears to be a finding that is generally valid for secondary IgG responses against soluble antigens (17) and for influenza virus in mice (2) and, variably, in guinea pigs (22). These results may be explained by primed T helper cells or primed B cells or both being CS-A resistant; the

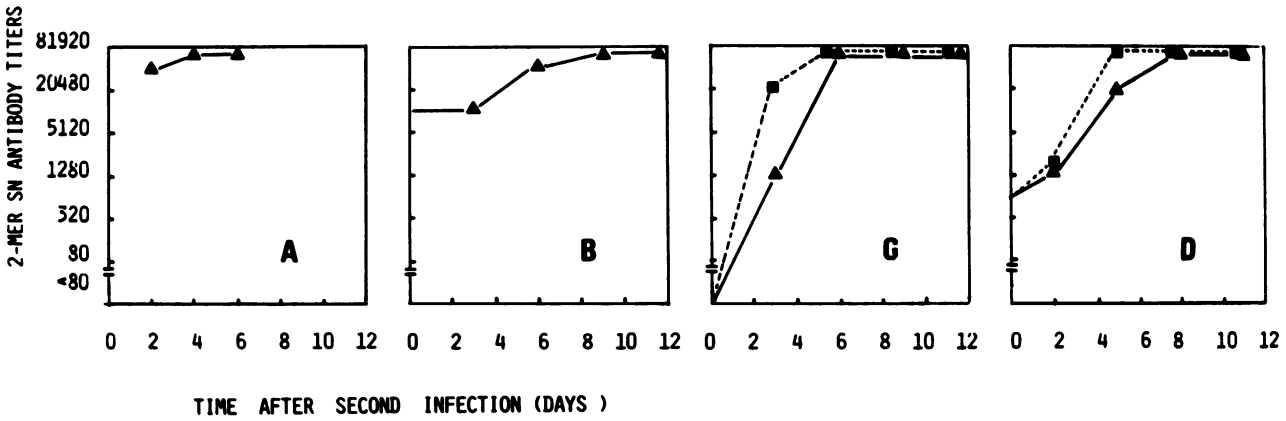

FIG. 5. Effect of CS-A on the secondary anti-VSV-Ind response. Four groups of mice primed with 10^6 PFU of virus 8 days (A) or 6 weeks (B) in advance, with 10^4 PFU 7 months (C) in advance, or with 10^8 PFU 15 months (D) in advance were challenged with 10^6 PFU of the homologous serotype together with CS-A treatment (▲) or without CS-A treatment (■).

present experiments cannot distinguish between these possibilities. The experimental evidence accumulated so far indicates that CS-A mainly influences T cells (6, 7, 30–34). Kunkl and Klaus (17) have in fact shown that primed carrier (protein antigen)-specific T cells are resistant to CS-A; thus, primed T helper cells are less susceptible to CS-A than are virgin T cells.

Interestingly, induction of secondary virus-specific cytotoxic T cells (CTL) in vitro and in vivo has been found to be rather susceptible to CS-A. If T help is necessary (2, 15) for CTL induction, a possibility that is not yet unequivocally proven, the obvious difference between CS-A-resistant T help requirements for secondary antibody responses compared with their presumed CS-A sensitivity for CTL induction suggests either different T helper mechanisms or other pathways of induction for CTL compared with that of B cells.

As has been shown for a variety of T-cell-dependent immune responses, CS-A must be given shortly before or together with the antigen to efficiently suppress the immune response (2, 15). In a primary anti-VSV response, once the switch to IgG had occurred, CS-A did not measurably impair the IgG response anymore. Since CS-A is assumed to exert its effect by inhibiting IL-2 synthesis or IL-2 receptor expression or both (10, 14, 21, 30), this result may suggest that IL-2 is not essential or is not the sole crucial B-cell growth factor during secondary responses (35); otherwise, some reduction of IgG responses may have been expected.

Although mice are not natural hosts for VSV, when infected i.c. with VSV, they usually died rapidly (within a few days), regardless of their immune status and of CS-A treatment. Preexistent antibodies probably cannot reach and neutralize the virus injected i.c.; once initiated, infection spreads rapidly before an immune response can protect the host. However, the susceptibility of mice to infection i.v. or subcutaneously (in the footpad) with VSV increased substantially under CS-A treatment. When i.v. infected mice were treated for 8 to 12 days with CS-A, they regularly developed paralysis, and some died after 14 to 16 days; control mice did not show signs of this disease. When injected with VSV into the footpad, CS-A-treated mice succumbed to paralytic disease, and mortality was high, whereas control mice had no disease or milder disease, and none died.

These findings suggest that, during CS-A treatment, i.v. injected VSV may efficiently trigger an unimpaired IgM response that may partially contribute to the control of VSV. If injected into the foot, VSV may trigger this B-cell response less well or infect peripheral nerves more readily or both. Since VSV does not usually replicate in mouse organs

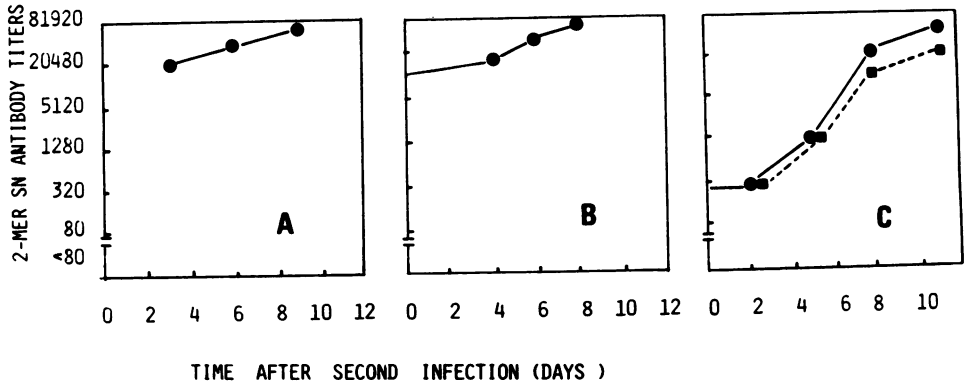

FIG. 6. Effect of CS-A on the secondary anti-VSV-NJ response. Three groups of mice primed with 10^6 PFU of virus 8 days (A) or 6 weeks (B) in advance or with 10^8 PFU 15 months (C) in advance were challenged with 10^6 PFU of the homologous serotype together with CS-A treatment (●) or without CS-A treatment (■).

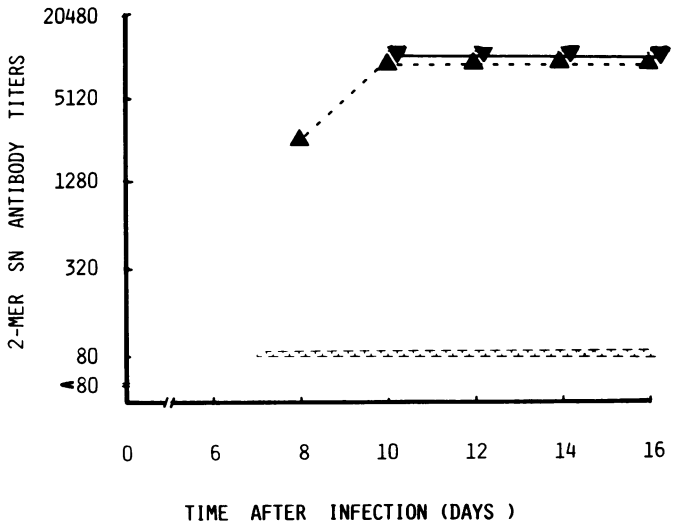

FIG. 7. Effect of CS-A on the preformed anti-VSV-Ind IgG SN antibody response. Mice primed with 10^6 PFU of VSV-Ind showed identical 2-MER SN antibody titers when CS-A-treated mice (CS-A from days 7 to 16) (▼) were compared with those of untreated control mice (▲). The hatched area represents the course of CS-A treatment.

other than the brain, infection of which leads to death (8, 9, 12), it is reasonable to assume that these mechanisms may enhance the chances of VSV reaching the brain either via blood or via nerves (8, 9, 29). The latter route of virus transport may be successful because the virus is protected from antibodies. The fact that i.v. injection of VSV into CS-A treated mice also results in about 50% lethality may be due to haematogenic spread to the brain or may be caused by viral infection of the tail nerves damaged by the injection needle (29). In the present study, we cannot determine whether impairment of antiviral CTL induction, impairment of T cells involved in delayed-type hypersensitivity, or impairment of T helper cells inducing IgG antibodies is responsible for increased susceptibility of mice to VSV. Since *H-2*^{k} mice fail to generate CTL against VSV but are not more susceptible to VSV injected i.c. or in the footpad

TABLE 1. Effect of CS-A treatment on survival after footpad inoculation of VSV-Ind[a]

Mouse strain	Virus dose (PFU)	Mean time to death (days ± SD)	No. of mice that died[b]
DBA/2 (*H-2*^{d})	10^7[c]	4 ± 2.9	5
	10^5[c]	5.6 ± 2.0	6
C57BL/6 (*H-2*^{b})[d]	10^7	8.6 ± 1.5	5
	10^5[c]	11 ± 2.2	6
B10.BR (*H-2*^{k})[e]	10^7	10.0 ± 0.6	6
	10^5[c]	11.8 ± 1.5	4

[a] CS-A treatment (60 mg/kg per day i.p.) was started on day −1 and continued daily up to day 10 of infection.

[b] In all groups six mice were infected.

[c] None of the mice that were not treated with CS-A developed paralysis or died.

[d] Four of six mice in the group receiving 10^7 PFU of VSV and no CS-A developed mild paralysis of hind feet on day 8, and two of them recovered by day 14, but none of them had died by the end of the observation period on day 20.

[e] One of six mice in the group receiving 10^7 PFU of VSV and no CS-A developed paralysis on day 7 and died on day 15 after inoculation in the footpad.

than are non-*H-2*^{k} mice as shown here and previously (23), the protective role of helper cells or T cells involved in delayed-type hypersensitivity and that of antibodies are probably crucial; we are currently attempting to evaluate the role of antibodies in B-cell-depleted mice.

CS-A has been in clinical use for several years; experience has shown that fewer infections occur during treatment than would be anticipated from experience with conventional immunosuppression (27, 32–34). Two points of clinical relevance should be stressed. First, VSV infection in mice documents the possibility that, under CS-A treatment, an otherwise mild infection with a virus for which mice are not natural hosts may become severe and lethal. Second, these and various other experiments also illustrate that vaccination creates CS-A-resistant antibody memory responses, yielding excellent protection against reinfection with viruses that are susceptible to antibody-dependent neutralization.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-17285 from the National Institutes of Health, by grant SNF 3.323-0.82 from the Swiss National Science Foundation, and by the Kanton Zürich. S.C. was supported by the Eidg. Stipendienkommission für Ausländische Studierende under the Indo-SWISS exchange programme.

We thank J. F. Borel, Biological and Medical Research Division, Sandoz Ltd., Basel, Switzerland, for the generous gift of CS-A; and Béatrice Borter and Rosina Caprez for their secretarial assistance.

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