

Effect of Immunosuppression on Recurrent Herpes Simplex in Mice

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Mice latently infected with herpes simplex virus were treated with immunosuppressive drugs either alone or combined with stimuli to the skin. Treatment with cyclophosphamide reduced spleen weights and severely depressed lymphocyte levels, but had no effect on healing after cellophane tape stripping (CTS) and did not affect the cutaneous hypersensitivity response after injection of inactivated herpes simplex virus. The drug, either used alone or combined with CTS, failed to increase the incidence of recurrent clinical disease, but increased the incidence of virus isolation after CTS. Prednisolone and azathioprine used together also reduced spleen weights and circulating lymphocyte levels. They slightly delayed healing after CTS, but had no effect on cutaneous hypersensitivity to herpes simplex virus. The treatment, either used alone or combined with CTS, slightly increased the incidence of recurrent clinical disease but did not increase the incidence of virus isolation after CTS. Treatment with antithymocyte serum severely depressed the levels of circulating lymphocytes and delayed the regression of HeLa cell tumors in mice. Used alone, the treatment slightly increased the incidence of recurrent clinical disease, but it failed to increase the incidence of recurrences after CTS. It increased the duration of recurrent herpetic lesions, although in uninfected mice healing after CTS was not affected. Silica altered the clinical course of primary infection with herpes simplex virus and increased the incidence of latency in the ganglia. It also delayed healing after CTS in uninfected mice, so it was not tested when recurrent herpes after CTS was assessed clinically. Treatment with silica alone did not increase the incidence of recurrent clinical disease or the incidence of virus isolation after CTS. The results demonstrate that potent immunosuppressive drugs are much less effective than simple cutaneous manipulation in inducing recurrent lesions, and thus argue strongly for the importance of local factors in the pathogenesis of disease.

Herpes simplex virus (HSV) becomes latent in the sensory ganglia after primary infection (23). In humans, clinical disease recurs at intervals near the site of primary infection (16). The primary disease may be readily produced in various laboratory animals (23), but until recently it was not possible to produce recurrent clinical disease in these animals. Such disease can be induced in the mouse ear by cellophane tape stripping (CTS) of the skin (12). However, the proportion of mice which show recurrent clinical disease averages only 30%, and to improve the model it would be desirable to increase this figure. It has been proposed that latency and reactivation of HSV are controlled by the immune system (5). Immunosuppression is a well-documented precipitating factor in other latent herpesvirus infections of humans such as cytomegalovirus and varicella-zoster virus (2, 21), and experimentally, recurrent herpes simplex has been produced by immunosuppression of mice either alone (29) or combined with other stimuli (14, 15).

We have combined stimuli to the skin with treatments which affect various aspects of immune responses: cyclophosphamide (CPA) severely affects the B-cell system (26, 28), but can also affect T-cells; antithymocyte serum (ATS) primarily kills thymocytes (17); and prednisolone and azathioprine (P + A), a combination of drugs commonly used successfully in human transplant patients to prevent rejection of grafts, inhibits both humoral and cellular responses (4). In addition, we have used silica since this is known to affect primary HSV infection (31) and might alter immune responses through its action on macrophages (1).

MATERIALS AND METHODS

Latently infected mice. Female, 4-week-old outbred Swiss white mice were injected subcutaneously in the ear with 6×10^4 or 3×10^5 plaque-forming units of HSV-1 strain SC.16 (13). Only mice that showed definite erythema or paralysis of the ear during primary infection were used for experiments on reactivation. At least 4 weeks elapsed between infection and experimental treatment, and mice whose right

ears were then erythematous were excluded (12).

Stimuli to the skin. The right ears of groups of mice were irradiated with ultraviolet light for 50 s (6, 10). Cellophane tape was applied six times to the ears of other groups of mice (12).

Severity of clinical disease. For assessing clinical signs during primary or recurrent disease after trauma or after the subcutaneous injection of killed HSV, the severity of erythema was given a score from a scale of 1 to 4.

Immunosuppression. Prednisolone (Koch-Light Ltd., Colnbrook, England) was suspended in 0.5% methylcellulose in PBS and mixed with an equal volume of azathioprine (Imuran, Wellcome Reagents Ltd., London, England) dissolved in deionized water. Mice were injected intraperitoneally with 0.5 ml of the mixture on days 1 and 3 of the experiment and with prednisolone alone on day 5. The doses given were 50 mg of azathioprine per kg and 60 mg of prednisolone per kg (P + A). Stimuli were applied to the ear on day 6 of the experiment.

For preparation of ATS, the thymuses were removed from about 30 4-week-old male mice and placed into 10 ml of medium 199. The tissue was disaggregated by pressing it through a metal gauze, and the suspension was filtered through a column of absorbent cotton wool. Erythrocytes were removed by centrifugation through Ficoll-Paque (Pharmacia Ltd., London, England) (7) and the thymocytes which remained in the supernatant fluid were washed twice. Each of three rabbits received $\sim 5 \times 10^6$ cells in each of 6 subcutaneous sites. Three inoculations were given at weekly intervals and the rabbits were bled 1 week after the final dose. Mice were injected subcutaneously with 0.35 ml of ATS on days 1-3, 5, and 7 of the experiment. Stimuli to the skin were given on day 6.

Silica was obtained from G. Turner (Lister Institute of Preventive Medicine, Elstree, Herts, England). It was autoclaved as a 100 mg/ml suspension in PBS. Mice were injected intraperitoneally with 0.5 ml of this suspension on days 1 and 2 of the experiment, and the skin was stripped with cellophane tape on day 3.

Mice were injected intraperitoneally with 0.5 ml per mouse (100 mg/kg) of 6 mg of CPA (Koch-Light Ltd.) per ml in PBS, and 1 day later, the skin was stripped.

In experiments involving assessment of recurrent clinical disease, mice were examined daily for various periods after CTS of the skin. When P + A was tested, this period was 17 to 24 days, for ATS it was 21 to 27 days, for silica 10 days, and for CPA 19 days.

Isolation of virus from ears of mice. The skin was scraped from the upper surface of the ear, ground in 0.4 ml of medium 199, and the resulting homogenate was inoculated onto Vero cells grown in 25-cm² plastic flasks (Sterilin Ltd., Teddington, England) (12).

Test for hypersensitivity to HSV. HSV was grown in HeLa cells in medium 199 containing 5% reconstituted human AB plasma and the cells were disrupted mechanically. For inoculating mice, the virus preparation was inactivated at 56°C for 30 min. Uninfected HeLa cells prepared in a similar manner were the control inoculum. Uninfected or latently infected mice previously treated with the drug under test or with diluent received 20 μ l of virus or control inoculum subcutaneously in the right pinna. Hyper-

sensitivity was assessed by observation of erythema 6, 24 and 48 h after injection of virus, and at the two later times by measuring the thickness of the ear with a dial caliper gauge (Pocotest A-02T, Carobronze Ltd., London, England).

Tests of immunosuppression. CPA and P + A were tested by measuring their effect on spleen weight and number of circulating lymphocytes.

ATS was tested by measuring its effect on the number of circulating lymphocytes, and on the ability of HeLa cells to produce tumors in mice (22). Groups of mice were given ATS, or phosphate-buffered saline as control as described above. On day 2 all mice were inoculated subcutaneously with 10^7 HeLa cells. The size of the resulting tumors was monitored for 3 weeks.

Treatment with silica was assessed by testing its effect on primary infection with HSV, and on the establishment of latent infection. Eight-week-old mice were injected subcutaneously in the ear with 6×10^5 plaque-forming units of HSV-1 1 day after two daily intraperitoneal doses of 50 mg of silica, and the subsequent clinical signs were scored for severity. In addition, 12 weeks later, the second, third, and fourth cervical ganglia were removed aseptically and cultured in 0.5 ml of growth medium for 4 days. They were then ground in Griffith tubes and samples of 50 μ l were inoculated onto monolayers of Vero cells to detect the presence of HSV.

RESULTS

Efficacy of immunosuppression. P + A reduced the mean spleen weight of groups of 5 mice by an average 15% for at least 10 days after the start of treatment. Blood lymphocyte levels were depressed on average by 50% for the same period. CPA reduced the spleen weight by 40 to 50% for 6 days after treatment. By day 9 the weight of the spleens of treated mice had increased above that of control mice, a result similar to that of other workers (15, 26), but the number of circulating lymphocytes remained depressed by 40 to 60% for at least 11 days (Fig. 1).

ATS depressed blood lymphocyte counts by 57 to 81% in a group of 10 mice from days 4 to 14 of treatment, a result comparable to those reviewed by Lance et al. (17). In such mice injected with HeLa cells, tumors were slower to develop than in control mice, but did not regress. A similar inhibition of the regression of HeLa cell tumors in mice treated with ATS was found by Stanbridge and Perkins (22) in their work on the assay of the efficacy of ATS.

The severity of clinical lesions in primary infection was affected by silica. From the day after infection until day 10, mice treated with silica showed less erythema in the ear. Thereafter, clinical signs were more severe in the group treated with silica. Twelve weeks after infection, HSV was isolated from the ganglia of 16 of 36 (44%) mice treated with silica and 9 of 35 (26%) control mice.

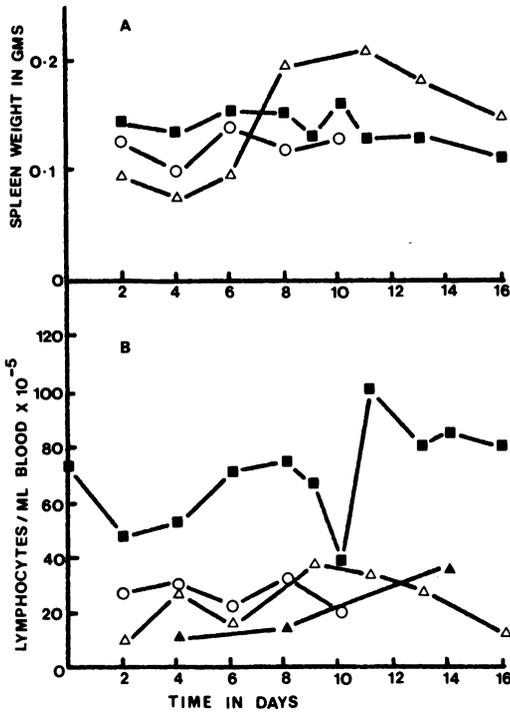


FIG. 1. (A) Effect of cyclophosphamide (Δ), prednisolone and azathioprine (\circ), or diluent (\blacksquare) on spleen weights. (B) Effect of cyclophosphamide (Δ), prednisolone and azathioprine (\circ), antithymocyte serum (\blacktriangle), or diluent (\blacksquare) on mean number of lymphocytes per ml of blood from five mice. See text for details of doses, etc.

Effect of immunosuppression on healing.

To test the effect of immunosuppression on healing, the right ears of groups of uninfected mice at least 7 weeks old were subjected to CTS 6 times. The mice were given either immunosuppressive drugs or diluent over the same time schedule, and the severity of clinical signs was recorded daily.

Both P + A and ATS slightly increased the severity of the reaction, and in 4 of 20 mice they prolonged erythema by about 3 days. However, this was not serious enough to interfere with clinical assessment of recurrent disease in latently infected mice. On the other hand, silica increased the severity of the reaction and prolonged erythema to such an extent that clinical assessment of recurrent disease was not possible when silica was used. Therefore, in mice treated with silica, reactivation of infection was assessed by isolation of virus from the ears.

CPA had no effect on the healing of damage due to CTS.

Effect of immunosuppression on hypersensitivity to HSV. As many immunosuppres-

sive drugs are also anti-inflammatory, it was necessary to see if they affected the cutaneous hypersensitivity reaction to HSV, which may well be a major component of the lesion of recurrent herpes simplex. This hypersensitivity was characterized by erythema which developed only in latently infected mice given inactivated HSV. The erythema was slightly more severe 6 h after inoculation of HSV than after 24 or 48 h.

In latently infected mice 24 and 48 h after injection of inactivated HSV, the inoculated ear was 50% thicker than the other ear. In latently infected animals given an uninfected HeLa cell preparation, or in uninfected animals given inactivated virus, ear thickness was not increased. Neither the ear thickening nor the erythema specific to latently infected animals given inactivated virus was affected by CPA given 4 days before challenge or treatment with P + A started 9 days before challenge.

Effect of immunosuppression on clinical recurrence of herpes simplex. CTS induced recurrent clinical disease in 43% of mice tested, and the erythema lasted, on average, 4.7 days. Immunosuppression with ATS or CPA did not alter the proportion of mice that developed recurrent disease, but with P + A the incidence was increased to 63%. However, this difference was not significant (χ^2 : $P > 0.05$). However, the duration of the erythema in clinical recurrences was significantly increased by immunosuppression with ATS (t -test: $P < 0.01$) although not with CPA or P + A (Table 1).

Immunosuppression with CPA or silica did not itself increase the incidence of clinical recurrence that occurs spontaneously (12), but with P + A or ATS the incidence was slightly above this background (Table 1).

Isolation of virus from ears. Since irradiation

TABLE 1. Recurrent clinical disease in latently infected mice

Treatment	No. of mice tested	Recurrences		Mean duration of erythema, days \pm SD ^a
		No.	%	
CTS	72	31	43	4.66 \pm 3.56
NRS ^c + CTS	32	14	44	5.53 \pm 3.08
ATS + CTS	53	20	38	9.04 \pm 6.87 ^b
P+A + CTS	46	29	63	6.19 \pm 2.96
CPA + CTS	26	11	42	7.27 \pm 5.80
ATS	27	3	11	4.5 \pm 1.87
P+A	45	4	9	9.25 \pm 7.5
CPA	30	1	3	3
Silica	30	1	3	3

^a SD, Standard deviation.

^b Significantly different from CTS alone (t -test: $P < 0.01$).

^c NRS, Normal rabbit serum.

tion with ultraviolet light induces recurrent disease in humans (30) and the appearance of infectious virus in the mouse ear (6), the right ears of groups of immunocompetent mice or mice immunosuppressed with ATS or P + A were irradiated for 50 s. Three days after irradiation, the animals were killed and the skin of the ears was tested for HSV. Virus was isolated from the ears of 18% of irradiated, immunocompetent mice, and this proportion was not increased by treatment with ATS or P + A. HSV was not isolated from the ears of 29 mice treated only with P + A; it was isolated from 1 of 15 animals given ATS (Table 2).

In other experiments, attempts to isolate virus were made at daily intervals after CTS of the skin of animals treated with silica or CPA (Table 3). HSV was not isolated after silica treatment alone and such treatment did not increase the proportion in which virus was found after CTS of the skin.

By contrast, on days 3-5 after CTS of the skin, virus was isolated from a significantly higher proportion ($P < 0.001$) of mice treated with CPA than from control animals.

To check whether erythema indicated the presence of infectious virus in the skin, the ears of mice were stripped and sampled either on the first day that new erythema appeared, or if erythema was continuously present, on the fourth day after CTS. Virus was isolated from 14 of 21 (67%) immunocompetent mice tested on the first day of erythema and from 3 of 8 (38%) of those tested 4 days after CTS. In mice treated with P + A, virus was isolated from 3 of 3 mice

on the first day of erythema, and from 2 of 9 (22%) sampled on the fourth day after CTS.

DISCUSSION

It is likely that controls of HSV latency exist at various sites, particularly in the sensory ganglia and in the skin or other peripheral tissue. These controls might well have different functions; those in the ganglion might limit virus production and thereby maintain latency, and those in the skin might limit active infection and thereby the chance of clinical disease. Controls at different sites might also involve different mechanisms which may or may not act through the immune system (3).

In some studies, immunosuppression failed to induce recurrent disease (6, 15, 24), and in others, disease developed in a small number of treated animals (17% in hairless mice treated with corticosteroids [29]; about 10% in the present study after P + A or ATS). This suggests that the immunosuppressive drugs tested affected only some of the control mechanisms, so that most animals remained free from disease. Since nearly 10% of clinically normal latently infected mice have virus in their skin at any time (T. J. Hill, D. A. Harbour and W. A. Blyth, J. Gen. Virol, in press), it may be these animals that develop clinical disease after treatment with P + A or ATS.

The lack of increased incidence of recurrent clinical disease when treatment with ATS or CPA is combined with stimuli to the skin cannot be interpreted as suggesting that immune mechanisms are not involved in control of latency. Furthermore, the short term immunosuppression used experimentally may not have the same effects as the long term treatment often used in humans. Stevens and Cook (25) and Lehner et al. (18) postulated that latent infection in the ganglion may be controlled by binding of immunoglobulin G to the surface of the infected neuron, and Costa et al. (8) showed that such binding markedly inhibits growth of HSV in Vero and Y-79 cells. The immunosuppressive treatments used in the present study primarily

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TABLE 2. Isolation of virus from the skin 3 days after irradiation of the ear with ultraviolet light

Treatment	No. of mice tested	Virus isolated	
		No.	%
UV ^a	44	8	18
P+A + UV	32	5	16
ATS + UV	15	2	13
P+A	29	0	0
ATS	15	1	7

^a UV, Ultraviolet light.

TABLE 3. Isolation of virus from skin after CTS of the ear

Treatment	No. of virus isolations on day after CTS ^a :					
	2	3	4	5	6	7
CTS	7/89 (8)	7/91 (8)	30/131 (23)	16/60 (27)	12/42 (29)	4/30 (13)
Silica	0/15	0/15	0/15	0/15	ND	ND
Silica + CTS	1/14 (7)	1/14 (7)	3/15 (20)	11/40 (28)	6/26 (23)	4/12 (33)
CPA	0/15	0/14	1/15 (7)	0/15	ND	ND
CPA + CTS	1/27 (4)	13/27 (48)	14/27 (52)	15/28 (54)	4/14 (29)	4/15 (27)

^a No. positive/no. tested. Numbers in parentheses are percent positive.

affect lymphocytes or macrophages, and in the short term would not affect the level of circulating antibody. Possible effects on the attachment of immunoglobulin G to cell surfaces are unknown.

The failure to increase substantially the incidence of recurrent disease when immunosuppression is added to CTS can be interpreted in various ways. The immune processes affected by the immunosuppressive drugs might be irrelevant to control of development of recurrent disease, although this is unlikely in view of the known importance of cell-mediated immunity in herpesvirus infections (3). Since infectious virus can appear in the ganglia during immunosuppressive treatments (19), an increased incidence should occur unless trauma itself induces disease with high efficiency and immunosuppression acts on controls at the same sites as trauma. Therefore, the lack of increased incidence may further suggest that trauma alone acts as both a skin and ganglion trigger (11). The low incidence of disease that results when immunosuppression is used alone suggests that the drugs release the infection from control less efficiently than trauma. There is some indication that treatment with P + A increases the incidence of clinical disease (63% as compared with 43% with trauma alone) which might suggest an action on control mechanisms not affected by trauma.

It is possible that trauma itself acts in part by local immunosuppression (11), since prostaglandins, which are known to be produced in skin after trauma or irradiation with ultraviolet light, inhibit antibody-dependent cell cytotoxicity of human fibroblasts infected with HSV (27).

Although immunosuppression did not affect the incidence of recurrent lesions induced by CTS, treatment with ATS, P + A, or CPA increased the duration of such lesions and with ATS this increase was statistically significant. Hurd and Robinson (15) also reported that in mice immunosuppression made the recurrent disease more severe after epilation. Furthermore, there are several reports of increased severity of herpes lesions in immunosuppressed humans, although there is disagreement whether the numbers of recurrences are increased (9, 30).

Treatment of mice with CPA did not increase the likelihood of isolating virus from the ear. However when such treatment was added to CTS of the ear, virus was isolated from a higher proportion of animals (about 50% on each of days 3-5 after CTS) than with CTS alone (about 25%) even though in other experiments the incidence of clinical disease was the same (42 to 43%) in animals with either treatment. In exper-

iments where attempts are made to isolate virus at set times after CTS, isolation is made less likely by the fact that recurrent lesions may develop 2 to 3 days before sampling the ear, so that at sampling, the virus may have been eliminated. The increased isolation from animals treated with CPA and CTS suggests that in such animals virus multiplies more (14, 20) or is eliminated from the tissue more slowly. Treatment with CPA can alter latent infection in the ganglion so that HSV can be isolated by inoculating disrupted tissue into cell cultures (19). In studies of mice infected in the cornea, T. J. Hill and K. Ahluwalia (unpublished data) found the same. However, this change does not result in clinical disease, which underlines the suggestion that control of the infection is likely to involve several mechanisms. Moreover, in the relatively huge doses used experimentally, CPA is likely to have effects other than immunosuppression (4).

Although treatment with silica neither induced recurrent lesions nor affected the incidence of lesions when combined with CTS, it increased the duration and severity of lesions in the primary HSV infection. It also delayed healing after CTS in uninfected animals. Treatment with silica before primary infection also increased the proportion of mice which became latently infected, perhaps by damaging macrophages (31) and thereby allowing more virus replication in the skin and a consequently increased supply of virus to the ganglion.

We conclude that immune mechanisms may be involved in the healing of recurrent herpes simplex but are probably not the only factors that control the latent infection.

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