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Immunomodulator-Induced Resistance Against Herpes Simplex Virus[1] (39730)

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Introduction. Viral infections can be inhibited by treatment of animals with a variety of immunomodulators (1-6). The mechanisms of antiviral action of immunomodulators, however, have been defined in only a few instances. The protective effects of polyriboinosinic-cytidylic acid and of tilorone hydrochloride appear to be due to the systemic production of high levels of interferon (5). Investigations have not been able to establish a similar role for interferon in the antiviral effects of synthetic polyanionic immunomodulators such as pyran and chlorite-oxidized carboxyamylose (COAM), but have suggested that cells of the activated reticuloendothelial system might be involved (1, 4). Pyran, as well as biologic immunomodulators such as *Mycobacterium bovis* strain BCG and the killed vaccine of *Corynebacterium parvum,* alter the reticuloendothelial system, causing enhancement of phagocytic clearance of particles and increased microbicidal activity (7). These immunomodulators also markedly enhance the capacity of the host to inhibit tumor growth, and the antitumor mechanism has been demonstrated to involve activated macrophages (8, 9). More recently, levamisole has been demonstrated to enhance resistance to viral infection, although the mechanism remains poorly defined (2). The present studies were undertaken to compare the antiviral activities of several of these diverse immunomodulators in two experimental herpes simplex virus models which were selected to provide a systemic infection (intraperitoneal route) and a local infection (intravaginal route) that is characterized by direct nerve root spread to the central nervous system (10). Experiments were also designed to investigate whether the immunomodulators would be effective in mice with impaired host resistance.

Materials and methods. Mice. Female Swiss mice obtained from Simonsen Laboratories, Gilroy, Calif. were used for all experiments.

Drugs. Corynebacterium acnes (ATCC 11828) was grown as previously described (11), the organisms were washed, suspended at 15 mg/ml wet weight, heat-killed at 60° for 1 hr, and stored at 4°. A killed vaccine of *C. parvum,* containing 7 mg/ml wet weight of organisms, was received from Burroughs-Wellcome, Research Triangle, N.C. Pyran (lot XA 124-177) was purchased from Hercules, Inc., Wilmington, Del., suspended in phosphate-buffered saline (PBS), and brought into solution by the addition of NaOH to pH 7.0. A 2.5% solution of oyster glycogen type II (Sigma Chemical Co., St. Louis, Mo.) was prepared in PBS and stored at 4°. Levamisole (R12564, Janssen Research and Development Co., New Brunswick, N.J.), lot 75A15/786, was obtained courtesy of Dr. Gerald Fisher, Tripler Army Medical Center, Honolulu, Hawaii, and dissolved in PBS. Silica No. 12, particle size between 2 and 10 nm, was obtained courtesy of Dr. Benkert, Dorentruper Sand-und Thonwerke BmbH, Dorentrup, West Germany. For some experiments, silica, 325 mesh (Sargeant-Welch Co.) was prepared as described by Selgrade and Osborn (12).

Virus and Cells. A pool of herpes simplex virus type 2 (HSV-2), MS strain, was prepared in primary rabbit kidney cells as previously described (10), and titered 5×10^6 plaque-forming units (PFU) on secondary mouse embryo fibroblasts (MEF). Studies of HSV-2 pathogenesis were performed as previously described (10). Fetal lamb kid-

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ney cells were used for titration of HSV-2 in organs. All medium consisted of 10% inactivated fetal calf serum, Eagle's minimal essential medium with Earle's balanced salt solution (EMEM), 100 units/ml of penicillin, and 50 μg/ml of streptomycin.

Antiviral studies in vivo. Swiss mice were prophylactically treated with the immunomodulators according to the following regimens: *C. acnes*, 150 mg/kg ip, Day −14; *C. parvum*, 70 mg/kg ip, Day −14; pyran, 25 mg/kg iv, Day −1 or 75 mg/kg ip, Day −1; and levamisole 1 mg/kg ip, Day −1. Controls included mice inoculated ip with glycogen (0.2 ml, Day −5) or PBS (0.2 ml, Day −14).

Mice were challenged ip with approximately 2×10^5 PFU of virus, or intravaginally (i.vag.) with approximately 1×10^5 PFU as previously described (10). Experimental groups of animals usually consisted of at least 15 mice. Animals were checked daily for mortality. The mean survival time was calculated and compared with that in control groups by the Student t test. The mortality in drug-treated as compared with control groups was evaluated using the χ^2 test with Yates' correction factor.

Results. Comparison of immunomodulators against ip and i.vag. HSV-2 infection in adult mice. Prophylactic systemic treatment (iv) of mice with pyran or treatment with corynebacteria or pyran at the site of virus inoculation (ip) significantly protected mice against lethal systemic infection with HSV-2 (Table I). In contrast, treatment by the same regimens did not significantly alter mortality of a local genital HSV-2 infection of mice, in which the virus spreads by direct nerve root extension to the central nervous system (10). The mean survival time of intravaginally infected mice was significantly increased, however, by systemic treatment with pyran. The results are similar to previous findings (13, 14) which have demonstrated that iv treatment of young adult BALB/c mice with pyran can significantly decrease the mortality of mice infected intravenously or intravaginally, but that the activity against the vaginal infection was less effective than against the systemic infection. Treatment with levamisole or glycogen was ineffective against either infection. In another experiment levamisole was administered twice, on the day prior to and the day of ip HSV-2 infection; this regimen was also ineffective (data not shown).

Treatment of suckling rats with levamisole has been reported to increase resistance to HSV-2 infection (2), and experiments were thus designed to determine the effectiveness of immunomodulators in young mice. Suckling mice averaging 8 days of age were treated ip with pyran or levamisole and infected ip with HSV-2 24 hr later. Levamisole had no protective effect on the course of the disease, as measured either by a reduction in mortality or prolongation of survival time. Treatment with pyran produced a slight reduction in mortality from 100% in controls to 75% ($P < 0.05$), and prolonged the survival time of mice from 6.0 ± 0.2 days in the control group to 9.8 ± 0.6 days in the pyran-treated group ($P < 0.01$). An attempt was also made to determine whether levamisole, in conjunction with *C.*

TABLE I. COMPARISON OF IMMUNOMODULATOR-INDUCED RESISTANCE AGAINST HSV-2 INFECTION IN ADULT MICE.[a]

	Infection ip		Infection i. vag.		
Treatment	Dead/Total	Percent	Dead/Total	Percent	Mean survival time ± SE
PBS	15/15	100	9/15	60	7.7 ± 0.2
C. acnes	3/15*	20	13/16	81	8.5 ± 0.4
C. parvum	4/14*	29	14/15	93	8.8 ± 0.3
Pyran ip	7/15*	47	12/15	80	10.9 ± 0.9
Pyran iv	8/15*	53	8/14	57	10.6 ± 0.6*
Levamisole	11/15	73	12/15	80	9.2 ± 0.4
Glycogen	11/15	73	9/15	60	8.2 ± 0.3

[a] Mice were treated with the immunomodulators as indicated in Materials and methods, inoculated ip or i. vag. with HSV-2, and the mortality and mean survival time of the mice that died were calculated.

* $P < 0.05$.

acnes treatment, could protect young mice against HSV-2 infection. Treatment of suckling mice with *C. acnes* alone was ineffective (16), and it was possible that levamisole treatment could enhance immunologic maturation processes. Treatment of 8-day-old mice with levamisole, *C. acnes* alone, or the combination of the two immunomodulators, however, had no protective effect on the course of disease in mice infected 14 days later (3 weeks old) with HSV-2. Prophylactic treatment of the 3-week old mice with pyran (75 mg/kg ip, −24 hr) was similar to treatment of suckling mice, causing a slight reduction in mortality and an increased mean survival time from 8.2 ± 0.4 to 12.2 ± 1.7 days ($P < 0.05$).

Effect of silica treatment on HSV-2 infection and immunomodulator-induced protection. If the antiviral action of the corynebacteria or pyran were mediated through macrophages, less protection might be evident in mice depleted of macrophages. Silica treatment, which alters a number of immunologic responses, is toxic for macrophages and transiently depletes the macrophage population (15, 20, 21). When differential counts were performed on peritoneal cells obtained 2–24 hr after ip silica treatment, it was apparent that the monocyte/macrophage and lymphocyte populations were decreased while the polymorphonuclear cell population was dramatically increased from 1−5% to approximately 50% of the population. Although there is considerable variation with the use of silica, treatment with either of two different preparations of silica prior to ip inoculation of HSV-2 consistently enhanced mortality to the disease at least 10-fold as compared to control animals (Table II).

TABLE II. EFFECT OF SILICA TREATMENT ON SYSTEMIC HSV-2 INFECTION.[a]

Experiments	$\log_{10}$ LD_{50}	
	Normal mice	Silica-treated mice
Experiment 1		
Sargeant-Welch silica	2.7	3.7
Dorentruper silica	2.7	3.7
Experiment 2		
Dorentruper silica	2.4	3.7

[a] Adult mice were treated ip with PBS, Sargeant-Welch silica (50 mg/kg), or Dorentruper silica (40 mg/kg) 2 hr prior to ip challenge with 10-fold dilutions of HSV-2, mortality was observed, and the LD_{50} doses per 0.2 ml of inoculum were calculated.

This increase in mortality was reflected in a change in the viral pathogenesis. When mice were inoculated ip with 2×10^3 PFU of HSV, 20% mortality occurred in the untreated control and 100% in the silica-treated group. Organs of groups of three mice from each group were obtained and pooled for assay at intervals during the course of the disease. In silica-treated mice, virus was present in all of the visceral organs 2 days after infection, with the highest titers in the spleen, kidney, and blood (Fig. 1). Virus persisted systemically until at least Day 4, at which time HSV-2 was present in both the spinal cord and brain stem. Progressive growth occurred in the central nervous system, with HSV-2 appearing in the cerebrum on Day 6. In the control group, low levels of virus were found sporadically in visceral organs, and virus did not appear in the central nervous system until Day 6. In untreated adult animals infected ip with a lethal dose (1×10^5 PFU) of HSV-2, virus also appears in the visceral organs late in the course of infection (Day 7) and primarily is in the spleen, lung, and kidney (Kern, unpublished observations).

The pathogenesis results indicated that it would not be appropriate to determine the protective effects of an immunomodulator in normal and silica-treated animals infected with the same dose of HSV-2, since the effective challenge in silica-treated mice would be greater than that in normal mice. Thus, mice were treated with the immunomodulators, then with PBS or silica, and challenged with 10-fold dilutions of HSV-2 in order to determine the effective LD_{50} in each group. Although silica treatment enhanced the LD_{50} of mice more than 100-fold, the immunomodulators were as effective in silica-treated mice as in normal mice (Table III). In fact, the LD_{50} of HSV-2 in *C. acnes*- or pyran-treated mice was almost the same in normal and macrophage-depleted mice.

Discussion. In this comparative study, adult mice were markedly protected from

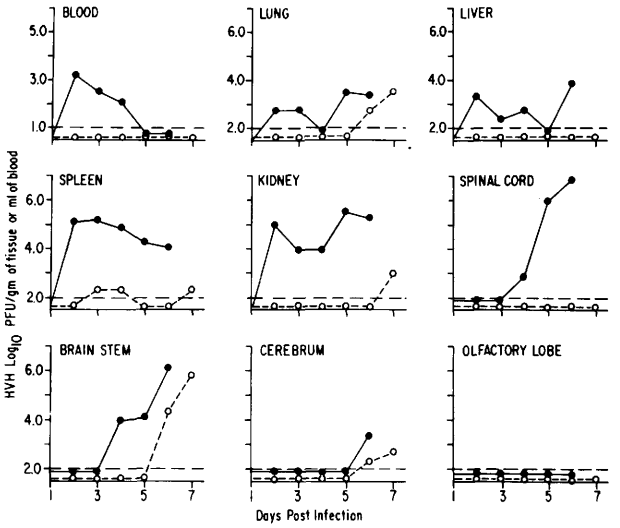

FIG. 1. The effect of silica treatment on the pathogenesis of herpes simplex virus (HVH) type 2 infection of mice. Virus titers in organs from silica-treated mice (●) or PBS-treated mice (○).

TABLE III. LACK OF EFFECT OF SILICA ON ACTIVITY OF C. ACNES OR PYRAN AGAINST HSV-2 Infection[a]

Immunomodulator treatment	PBS		Silica	
	$\log_{10}$ LD_{50}	Protection ($\log_{10}$)	$\log_{10}$ LD_{50}	Protection ($\log_{10}$)
PBS	2.3		4.7	
C. acnes	0.9	1.4	1.0	3.7
Pyran iv	1.4	0.9	1.7	3.0

[a] Groups of 15 mice each were treated ip with PBS or *C. acnes* (150 mg/kg) 14 days prior to, or with pyran (25 mg/kg iv) 1 day prior to virus challenge. Mice were treated with silica (40 mg/mouse ip) or PBS 2 hr prior to ip challenge with tenfold dilutions of HSV-2 ranging from $10^{-0.7}$ to $10^{-5.7}$.

intraperitoneal infection with HSV-2 by administration of pyran or killed corynebacteria, similar to data reported separately for *C. acnes* and pyran (13, 16). While both agents were effective against ip HSV-2 infection, only pyran significantly increased the survival time of mice infected intravaginally. It is of interest that prophylaxis with another immunomodulator, *Mycobacterium bovis* strain BCG, had no effect on mortality of mice from intravaginal HSV-2 infection (17). The results suggested that the antiviral effect of corynebacteria against HSV-2 might be exerted locally rather than systemically. Local administration of *C. parvum* appears to be more effective than does systemic administration against tumor growth (8), while the antitumor and antimicrobial action of pyran is expressed both locally and systemically (9). The differences between the two immunomodulators may be related to the induction by *C. parvum* of local granulomas with activated macrophages (8), while granuloma formation is not prominent after pyran administration (18).

Treatment of either adult or suckling mice with the immunomodulator levamisole was ineffective. These results are in contrast to those reported by Fisher *et al.* (2), who demonstrated marked protection of suckling 10-day-old rats from HSV-2 infection. Starr *et al.* (6) have also recently reported the ineffectiveness of levamisole, typhoid vaccine, brucella vaccine, or staphage lysate against HSV-2 infection in suckling mice. Only prophylactic BCG treatment was able to modify the survival rate of young mice. The mechanism of antiviral action of BCG and pyran in young mice remains to be defined.

Experiments were performed in an effort to determine whether activated macrophages were involved in the antiviral resistance produced by pyran and the corynebacteria. Zisman *et al.* (19) and DuBuy (20)

have demonstrated that adult mice can be made more susceptible to HSV infection if the mice are treated with silica, which is selectively toxic for macrophages (21). The present experiments with silica established the degree of enhanced susceptibility; the LD_{50} in silica-treated mice was consistently increased 10-fold or more over that in controls. Our results confirm and extend the observations of Zisman *et al.* (19) on pathogenesis of the infection. In silica-treated mice, virus replication in the visceral organs was significantly enhanced. The infection was associated with a viremia, earlier seeding of the central nervous system, and a shorter survival time than in untreated mice. Although silica treatment profoundly altered the course of the disease, animals receiving the immunomodulators pyran or *C. acnes* were protected as effectively as normal mice. These data would suggest that macrophages or other possible immunologic components affected by silica (15, 21) are not the critical determinant in the protective effects of the immunomodulators. However, the immunomodulator treatments cause an influx of macrophages into the peritoneal cavity and an hepatosplenamegaly associated with increased reticuloendothelial capacity (7, 8), which could decrease the effectiveness of silica. Immunomodulators such as *C. parvum* have also been demonstrated to increase macrophage colony formation from stem cells in the bone marrow (22). If the immunomodulators can amplify the residual reserves of cells, it may be impossible to deplete mice sufficiently of macrophages.

Results of pathogenesis studies have suggested that the antiviral effects of these immunomodulators are exerted during the first phases of the viral infection and do not involve enhancement of specific immunologic responses, but rather involve nonspecific resistance factors (14, 16). Previous results with pyran have demonstrated that the drug was as effective in immunosuppressed mice deficient in T-cell function as it was in normal mice (23). The current results indicate that the pyran and *Corynebacterium* immunomodulators were also effective in mice markedly deficient in host resistance functions due to silica treatment. Further studies on the mechanism of action of these substances are currently underway. It is clear that certain immunomodulators have the potential for ameliorating virus infections that are exacerbated as a result of immunosuppression.

Summary. Adult mice were protected from mortality after intraperitoneal infection with herpes simplex virus type 2 by prophylactic treatment with the immunomodulators, *Corynebacterium parvum, Corynebacterium acnes*, or pyran. Treatment with pyran, but not with the corynebacteria, also increased the survival time of mice after local genital (intravaginal) infection with the virus. Treatment with levamisole or glycogen was ineffective against either virus infection. Levamisole and *C. acnes* were also ineffective against herpes simplex virus infection in suckling and weanling mice, while pyran was slightly effective. Silica treatment, to suppress macrophage function *in vivo*, increased the susceptibility of mice to herpes simplex infection by 10- to 100-fold. The increased susceptibility induced by silica was associated with early, sustained viral replication in the visceral organs. Although silica treatment markedly suppressed host resistance to herpes virus, this treatment did not inhibit the antiviral activity of pyran or *C. acnes*.

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1. Billiau, A., Muyembe, J. J., and deSomer, P., Nature N. Biol. **232,** 183 (1971).
2. Fischer, G. W., Podgore, J. K., Bass, J. W., Kelley, J. L., and Kobayashi, G. Y., J. Infec. Dis. **132,** 578 (1975).
3. Kern, E. R., Glasgow, L. A., and Overall, J. C., Jr., Proc. Soc. Exp. Biol. Med. **152,** 372 (1976).
4. Morahan, P. S., Regelson, W., and Munson, A. E., Antimicrob. Agents Chemother. **2,** 16 (1972).
5. Stringfellow, D. A., Overall, J. C., Jr., and Glasgow, L. A., J. Infect. Dis. **130,** 470 (1974).
6. Starr, S. E., Visintine, A. M., Tomeh, M. O., and Nahmias, A. J., Proc. Soc. Exp. Biol. Med. **152,** 57 (1976).
7. Munson, A. E., Regelson, W., Lawrence, W., and Wooles, W. R., J. Reticuloendothel. Soc. **7,** 375 (1971).
8. Woodruff, M. F. A., Transplant. Proc. **7,** 229 (1975).
9. Morahan, P. S., and Kaplan, A. M., Int. J. Cancer **17,** 82 (1976).

10. Overall, J. C., Jr., Kern, E. R., Schlitzer, R. L., Friedman, S. B., and Glasgow, L. A., Infect. Immun. **11,** 476 (1975).
11. Fischbach, J., and Glasgow, L. A., Infect. Immun. **11,** 80 (1975).
12. Selgrade, M. K., and Osborn, J. E., Infect. Immun. **10,** 1383 (1974).
13. Morahan, P. S., and McCord, R. S., J. Immunol. **115,** 311 (1975).
14. Breinig, M. K., Cline, P. F., and Morahan, P. S., Abstracts Annual Meeting A. S. M., p. 78 (1976).
15. Miller, S. D., and Zarkowe, A., J. Reticuloendothel. Soc. **19,** 47 (1976).
16. Glasgow, L. A., Fischbach, J., Bryant, S. B., and Kern, E. R., J. Infect. Dis., in press.
17. Baker, M. B., Larson, C. L., Ushijima, R. N., and Anderson, F. D., Infect. Immun. **10,** 1230 (1974).
18. Snodgrass, M. J., Morahan, P. S., and Kaplan, A. M., J. Nat. Cancer Inst. **55,** 455 (1975).
19. Zisman, B., Hirsch, M. S., and Allison, A. C., J. Immunol. **104,** 1155 (1970).
20. DuBuy, H., Infect. Immun. **11,** 996 (1975).
21. Allison, A. C., Harington, S. S., and Berbeck, M., J. Exp. Med. **124,** 141 (1966).
22. Dimitrov, N. V., Andre, S. Eliopoulos, G., and Halpern, B., Proc. Soc. Exp. Biol. Med. **148,** 440 (1975).
23. McCord, R. S., Breinig, M. K., and Morahan, P. S., Antimicrob. Agents Chemother. **10,** 28 (1976).

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