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SHORT COMMUNICATION

A DETERGENT-SOLUBLE EXTRACT OF VIRUS-INFECTED CELLS FREE FROM INFECTIOUS VIRUS PROTECTS MICE FROM LETHAL HERPES SIMPLEX VIRUS INFECTION

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SUMMARY Lethal infection with herpes simplex virus types 1 (HSV-1) and 2 was effectively prevented by previous immunization with a detergent-soluble extract (DSE) of virus-infected cells free from infectious virus without any adjuvant. This protective immunity seemed to last for at least one month. Neutralizing antibodies were elicited in mice immunized with DSE, but at a lower level than in animals immunized with live or killed virus. DSE did not protect athymic nude mice from death by HSV-1 infection, suggesting that a T cell-mediated immune response plays a major role in the protection.

Herpes simplex virus (HSV) is a pathogenic agent that preferentially affects organs of ectodermal origin. It causes life-long latency in humans and recurrent infection has frequently caused serious problems in various countries of the world. Since the promising trial by Price et al. (1975), several attempts have been made to develop effective and safe subunit vaccines (Zaia et al., 1975; Cappel, 1976; Kitces et al., 1977). Similar investigations have also been made on other herpesviruses, such as Marek's disease virus (Kaaken and Dietzschold, 1974; Lesnik and Ross, 1975), herpesvirus saimiri (Pearson and Scott, 1977) and Epstein-Barr virus (Thorley-Lawson, 1979). In the present work, we found that lethal HSV types 1 (HSV-1) and 2 (HSV-2) in mice could be prevented by previous immunization with a detergent-soluble extract of virus-infected cells free from infectious virus, and that a T cell-mediated immune response was involved in the protection.

CV-1 cells derived from African green monkey kidney were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS). The viruses used in the study were the Fukuda strain, the GC^+ variant of the Miyama strain (Nii and Kamahora, 1961), and the HF strain of HSV-1, all of which were kindly supplied by Dr. S. Nii, Okayama Universty, Japan,

and the UM268 strain of HSV-2, which was kindly supplied by H. Nishiura, Osaka University, Japan. These viruses were propagated in CV-1 cells and stocked at −70°C until use. Virus infectivity was assayed by the usual plaque titration technique on CV-1 cell monolayers in 24 mm tissue culture plates (Linbro, New Haven, Conn.). In brief, 0.2 ml of an appropriately diluted virus suspension was adsorbed for one hour at 37°C. Then overlay medium (MEM, 5% CS and 0.5% agarose) was added and plates were incubated for 3 days at 37°C in a humidified incubator containing 5% CO_2. Cultures were then fixed with 10% formalin for one hour. After removal of the overlay, plaques were stained with 0.3% methylene blue and counted. The virus titers of the Fukuda, Miyama, HF and UW268 strains were 5×10^7, 5×10^6, 5×10^7 and 2×10^7 PFU/ml, respectively.

Immunizing antigen was prepared as described by Lesnik and Ross (1975) with a slight modification. Confluent monolayers of CV-1 cells in roller bottles were infected with HSV-1 (Fukuda strain) at 37°C until the cytopathic effect became generalized. Then the medium was decanted, and infected cells were scraped off the glass and washed twice with phosphate-buffered saline (pH 7.4, PBS) by centrifugation at 700 *g* for 10 min. The pellet was resuspended in PBS at 4×10^7 cells/ml and sonicated for 3 min at 4°C (Insonator 200M, 9 kHz, Kubota Industries, Tokyo, Japan), followed by treatment with 0.5% (v/v) of Nonidet P-40 (Shell Chemicals U. K. Ltd., London, SEI) for 30 min at 0°C. The extract was then centrifuged at 3,000 *g* for 10 min and the supernatant was further centrifuged in an SW 50.1 rotor at 35,000 rpm for one hour at 4°C. Two-third of the supernatant was carefully aspirated and extensively dialyzed against PBS containing 1 g/liter of Bio-beads SM-2 (Bio-Rad Laboratories, Richmond, Calif.) for 48 h at 4°C to remove the detergent (Holloway, 1973). The final preparation, tentatively designated as DSE (detergent-soluble extract of virus-infected cells), did not contain any infectious virus detectable in tests on CV-1 cell monolayers for 3 weeks by blind passages. HSV-2 DSE was prepared by the same method as described above using the UW268 strain for inoculation. The protein content of DSE was measured by the method of Lowry et al. (1951).

Six-week-old male ICR mice (Japan Clea Laboratory, Tokyo, Japan) were immunized twice, with one week between immunizations, with HSV-1 DSE (0.5 mg·protein/mouse/immunization) by either the subcutaneous or intraperitoneal route. One week after the second immunization, mice were challenged with live virus by the same route as used for immunization. For challenge on the skin, the hair in an area of 2 cm×2 cm on one flank was removed. The next day, three circular scarifications were made on the area with a 2 mm-diameter trephine, and a cotton swab saturated with undiluted HSV-1 (Fukuda strain) was rubbed over the scarified area for a few seconds. For intraperitoneal challenge, an appropriate dose of HSV-1 (Miyama strain) was injected into the peritoneal cavity. For experiments using HSV-2, mice immunized with HSV-2 DSE were challenged with HSV-2 (UW268 strain) in the same way. For comparison of immunizing efficacies of DSE of the Fukuda strain and the full HF strain, which is avirulent when challenged extracerebrally, mice were immunized subcutaneously twice with live or formalin-inactivated HF strain (1×10^7 PFU/mouse/immunization) and similarly challenged with HSV-1 (Fukuda strain). Challenged mice were observed daily for 2 weeks, monitoring the appearance of herpetic lesions, and death from both types of infection. As shown in Table 1, unimmunized mice usually developed paralysis and died within a week when challenged with live virus, while significant numbers of immunized mice survived both HSV-1 and HSV-2 infection. The efficacy of vaccination with live or killed virus was confirmed as expected from the results of Price et al. (1975) and Kitces et al. (1977). Immunization with detergent-soluble

TABLE 1. *Efficacy of previous immunization against lethal HSV infection in ICR mice*

	Immunizing antigen used (dose)	Route of immunization and challenge	Virus challenge	Incidence of dermatitis (no. of mice with dermatitis/ no. of mice examined)	Mortality (no. of mice dead/no. of mice examined)
HSV-1	HSV-1 DSE[a] (0.5 mg)	intraperitoneal	Miyama 100 LD_{50}[b]		0/7
			50		0/8
			5		0/8
	None		100		6/7
			50		8/8
			5		6/8
	HSV-1 DSE (0.5 mg)	subcutaneous	Fukuda (undiluted)[c]	2/9	0/9
	None			9/9	9/9
	Live HF (10^7 PFU)			0/8	0/8
	Killed HF (10^7 PFU)			0/8	0/8
HSV-2	HSV-2 DSE (0.5 mg)	intraperitoneal	UW268 100 LD_{50}[d]		1/8
	None				8/8
	HSV-2 DSE (0.5 mg)	subcutaneous	UW268 (undiluted)[e]	2/7	1/7
	None			7/7	6/7

[a] DSE = detergent-soluble extract of virus-infected cells.
[b] LD_{50} of Miyama strain in 8-week-old ICR mice, 2.7×10^4 PFU/mouse.
[c] Undiluted Fukuda strain has a virus titer of 5×10^7 PFU/ml.
[d] LD_{50} of UW268 strain in 8-week-old ICR mice, 3.0×10^3 PFU/mouse.
[e] Undiluted UW268 strain has a virus titer of 2×10^7 PFU/ml.

extract of uninfected cells had no protective effect (data not shown).

Next we examined the persistence of the protective immunity by DSE. For this, ICR mice were immunized subcutaneously twice with HSV-1 as described above and challenged with HSV-1 (Fukuda strain) one to four weeks after the second immunization. As shown in Table 2, the immunity seemed to last for at least one month.

To see if the protective immunity by DSE was due to increase in serum-neutralizing antibody, we measured the antibody titers of immunized mice one week after the second immunization. The immunizing antigens used in this experiment were DSE of HSV-1 and HSV-2 with live and killed HF strain (1×10^7 PFU/mouse) for comparison. Volumes of 0.5 ml of serially diluted, heat-inactivated sera were incubated with an equal volume of virus suspension (1,000 PFU/ml) for 30 min at 37°C and then 0.2 ml samples of the mixtures were assayed for PFU on CV-1 cell monolayers as described above. Neutralizing antibody titers were expressed as the reciprocal of the dilution causing 50% plaque-reduction. As shown in Fig. 1, HSV-1 DSE constantly elicited neutralizing antibodies, but their titers were lower than those of mice immunized with live or killed HF strain. HSV-2 DSE induced a slightly lower level of neutralizing antibodies than HSV-1 DSE.

The dependence of the immunity on T cells was examined by subcutaneous immunization of femal BALB/c heterozygote (nu/+) and congenitally athymic nude (nu/nu) mice with HSV-1 DSE and then challenge with HSV-1 (Fukuda strain) as described above.

TABLE 2. *Persistence of protective immunity induced by DSE against cutaneous HSV-1 infection*[a]

Interval between the last immunization and challenge (week)	Immunizing antigen used (dose)	Incidence of dermatitis (no. of mice with dermatitis/no. of mice examined)	Mortality (no. of mice dead/no. of mice examined)
1	HSV-1 DSE (0.5 mg)	1/8	0/8
	None	8/8	8/8
2	HSV-1 DSE (0.5 mg)	0/8	0/8
	None	7/8	7/8
3	HSV-1 DSE (0.5 mg)	0/8	0/8
	None	6/8	6/8
4	HSV-1 DSE (0.5 mg)	1/8	1/8
	None	8/8	8/8

[a] Eight-week-old ICR mice were immunized twice with HSV-1 DSE and challenged with HSV-1 subcutaneously at the indicated time after the last immunization.

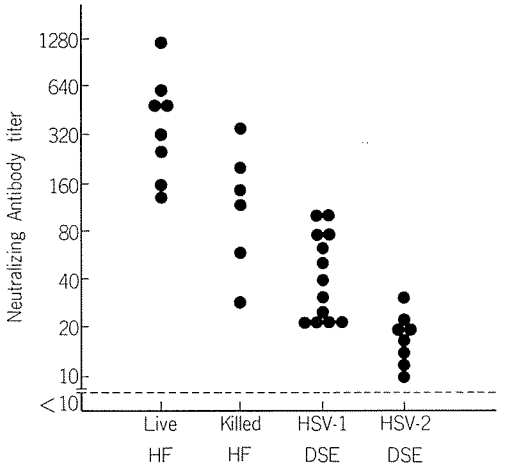

FIGURE 1. Comparison of serum-neutralizing antibody titers in mice immunized with live and killed HF strain, and HSV-1 and HSV-2 DSE.

As shown in Table 3, none of the athymic nude mice survived, but all the heterozygote mice survived.

Once primary infection occurs, humans harbor HSV in a latent form throughout life and reactivated HSV often causes a variety of troublesome diseases. Therefore, it seems reasonable to consider that HSV infection should be immunologically controlled by vaccination. Because of the latent and oncogenic potential of HSV, recent studies have been focused on subunit vaccines free from infectious virus and viral DNA. Cappel (1976) reported successful protection of rabbits from lethal HSV-1 infection by a subunit vaccine containing viral envelope proteins. Kitces et al. (1977) also reported significant protection of mice by DNA-free vaccine. Although our preparation was not completely free from viral DNA, it was effective in preventing HSV infection in mice. The immunity seemed to last for at least one month. An advantage of DSE is that it can elicit the protective immunity without the use of adjuvant. Moreover, DSE significantly blocks the establishment of trigeminal ganglionic latency subsequent to HSV-1 infection of guinea pig cornea (Ohashi, et al., 1980).

There have been many reports on the mechanism of resistance to HSV infection. But it is still uncertain whether antibody alone can prevent primary HSV infection, although a recent report (Davis et al., 1979) suggested that the effectiveness of antibody against HSV-1 infection was due not only to virus-neutralization, but to co-operation with other effector cell population. The involvement of cell-mediated immunity in recovery from HSV-1 infection was clearly shown by adoptive cell-transfer studies (Ennis, 1973; Oakes, 1975;

TABLE 3. *Efficacy of previous immunization against lethal cutaneous HSV-1 infection in heterozygote and athymic nude mice*[a]

Mice examined	Immunizing antigen used (dose)	Incidence of dermatitis (no. of mice with dermatitis/no. of mice examined)	Mortality (no. of mice dead/no. of mice examined)
BALB/c (nu/nu)	HSV-1 DSE (0.5 mg)	11/11	11/11
	None	11/11	11/11
BALB/c (nu/+)	HSV-1 DSE (0.5 mg)	2/9	0/9
	None	9/9	9/9

[a] Female BALB/c heterozygote (nu/+) and congenitally athymic nude (nu/nu) mice were immunized twice with HSV-1 DSE and challenged with HSV-1 by the subcutaneous route.

Rager-Zisman and Allison, 1976). Our results on athymic nude mice strongly suggest that T cell-mediated immune response plays an essential role in the protection. But we cannot yet decide which of the two mechanisms, humoral or cell-mediated immunity, plays the main role in defense elicited by DSE, because T cells are required for the production of anti-HSV antibody (Burns et al., 1975). Nevertheless, the fact that cellular immunity against HSV-1 can be induced by subunit vaccines (Zaia et al., 1975; Cappel, 1976) suggests that effectors other than antibody participate in the antiviral defense by DSE. Further investigations on this problem, including adoptive cell-transfer experiments, are now in progress.

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