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Tolerance and Suppression of Immunity to Herpes Simplex Virus: Different Presentations of Antigens Induce Different Types of Suppressor Cells

RACHEL D. SCHRIER,[1]† LEWIS I. PIZER,[1] AND JOHN W. MOORHEAD[2]*

Department of Microbiology and Immunology[1] and Department of Medicine,[2] University of Colorado Health Sciences Center, Denver, Colorado 80262

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In this report, we examine tolerance (hyporesponsiveness) and suppression of delayed hypersensitivity (DH) to herpes simplex virus (HSV) in mice, using two different forms of tolerogen: HSV particles and HSV-infected spleen cells. The intravenous injection of mice with either HSV particles or spleen cells 7 days before subcutaneous immunization with virus induced a profound state of unresponsiveness. This unresponsive state was mediated, at least in part, by suppressor T cells (T_s), which were demonstrated by passive transfer to naive recipients. However, different types of T_s were induced depending on the form of the tolerogen. The injection of HSV particles induced T_s which suppressed the induction but not the expression of DH. On the other hand, the injection of HSV spleen cells induced two types of T_s: one which inhibited the induction of the DH response and one which inhibited the expression of DH to HSV. Both tolerance and T_s are virus specific (i.e., the DH response to an unrelated virus was not inhibited) but not type specific for HSV type 1 and HSV type 2. Since both virus particles and virus-infected cells may be present in the blood during HSV infection, the induction of this type of immune regulation may influence the outcome of both acute and latent HSV infections.

The regulation of the immune response has been the subject of intensive study in recent years. However, relatively little is known concerning the in vivo negative regulation of the host immune response to a pathogen such as a virus. In contrast to most experimental antigens, a virus infects cells according to its tropism and mediates the insertion of viral proteins into the host cell membrane, thereby influencing the context in which they are seen. Virus infections have the potential to activate various limbs of the host immune response, some of which may provide an effective defense, whereas others may be ineffective or immunopathogenic. Specific regulatory mechanisms can also be activated, and the balance of these various responses determines the outcome of the infection.

Our laboratory has been studying delayed hypersensitivity (DH) to herpes simplex virus (HSV) in mice and the role of this response in protection against HSV infection. Numerous studies have shown that although HSV infections result in a substantial production of antibody in humans and experimental animals, this antibody seldom affords complete protection in acute infections and does not prevent recurrent infections (7, 24, 25, 29). On the other hand, T cells have been shown to provide protection in acute HSV infections, and the impairment of T cell activity increases the frequency and severity of recurrent infections (1, 7, 17, 24, 27–29, 31). The nature of the effector T cell(s) which mediate the protective response is not clear. Cytotoxic T cells directed against HSV-infected target cells are difficult to demonstrate directly from an infected mouse. Generally, the pretreatment of the infected host with cyclophosphamide or a culture of immune cells in vitro for 3 days before assay is necessary to demonstrate significant cytotoxic activity (9, 26). The protective value of this cytotoxic T cell response in vivo is unknown. Howes et al. (7) have reported that cells from HSV-immune donors which share major histocompatibility complex I region determinants with recipient mice can transfer long-term protection against HSV infection whereas those sharing major histocompatibility complex K, D antigens give short-term protection. Recent studies by Nash et al. (22) have shown that cells capable of transferring DH to HSV are able to reduce the virus titer in a local infection. The transfer of DH required IA subregion sharing between donor and recipient, although a significant reduction of local virus titers was only

† Present address: Scripps Clinic, La Jolla, CA 92037.

observed when donors and recipients were compatible across the entire major histocompatibility complex.

Our previous report characterized DH to HSV in mice and demonstrated that DH can be readily induced by the subcutaneous (s.c.) injection of virus (30). To expand our understanding of host-virus interactions, we examined the regulation of this particular response. This report characterizes tolerance (hyporesponsiveness) and the suppression of DH to HSV induced by the intravenous (i.v.) injection of two forms of the virus: HSV particles and HSV-infected syngeneic spleen cells (HSV-SC).

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from the American Medical Center, Lakewood, Colo., and Cumberland View Farms, Cumberland, Tenn.

Virus. HSV type 1 (HSV-1) strain KOS Ts5, a temperature-sensitive mutant that does not replicate in mice, was used in these experiments. This mutant is able to infect cells and produce proteins, but a defect in assembly blocks the production of infectious particles. The HSV-2 strain used was 186 (wild type). The growth and preparation of HSV stocks have been previously described (30). Briefly, stocks of HSV-1 (KOS Ts5) and HSV-2 (186) were grown in both rabbit skin cells and murine 3T6 cells (of BALB/c origin). The mice were immunized with virus grown in rabbit skin cells and challenged with homologous virus grown in 3T6 cells. The purpose of this protocol was to avoid the measurement of any response to cellular antigens as some cellular components were present in our virus preparations. Titers of virus stocks were determined by plaque assays. To avoid mortality, HSV-2 186 was UV inactivated for 10 min before use as an immunogen. Both HSV-1 (KOS Ts5) and HSV-2 (186) were UV inactivated for 10 min before use for challenge (see below). This served to reduce the nonspecific toxic effects at the skin test site of nonimmunized controls but did not affect the response in immunized mice.

Vesicular stomatitis virus (VSV) (strain Indiana) was grown in baby hamster kidney (BHK) cells. Confluent cell monolayers were infected at a multiplicity of infection of 0.1 PFU/cell in serum-free medium. Twenty-four hours later, when all cells showed cytopathic effects, the medium was removed and centrifuged at 200 × *g*. The supernatant was stored at −70°C, and virus titers were determined by plaque assay. All VSV preparations were UV inactivated for 30 min before use.

Induction and elicitation of DH to HSV. Mice were immunized by the injection of 4×10^6 PFU (HSV-2 and VSV were UV inactivated) s.c. over each limb. Six days later, the DH response was elicited by injecting 10^6 PFU of UV-inactivated virus in a volume of 10 μl into the dorsal side of the mouse ears. Ear swelling was measured 24 h later with an engineering micrometer (Mitutoyo, Tokyo, Japan). Results are expressed as increased ear swelling (δ), i.e., ear swelling of immunized mice minus ear swelling of control mice, in units of 10^{-4} in (ca. 2.54 nm). In all experiments, the ear swelling of control mice was less than 10 U.

Induction of tolerance of DH to HSV. The tolerance of DH to HSV was induced with two different forms of the virus. In one case, titers of the virus stock (grown in rabbit skin cells [29]) were determined, and 10^8 PFU was injected i.v. in a volume of 0.5 ml. This preparation is referred to as HSV-1 particles. The second form of tolerogen was HSV-SC. Normal spleen cells were infected with HSV-1 (KOS 5) at a multiplicity of infection of 10 to 20 PFU/spleen cell in 1 to 2 ml for 1 h at 37°C. RPMI 1640 (3 ml) supplemented with 0.5% normal mouse serum and 0.1 mM arginine was added, and the cells were incubated at 34°C for 12 h. The cells were washed extensively to removed unadsorbed virus and adjusted to 100×10^6/ml. Cells (0.5 ml) were injected i.v. into each mouse. In all experiments, the tolerogen was injected 7 days before the s.c. injection.

Transfer of tolerance. Single-cell suspensions of spleen and lymph node were prepared from mice injected i.v. with HSV particles or HSV-SC 7 days previously. The cells were mixed 1:1 and adjusted to 2×10^8 cells per ml, and 0.5 ml was injected i.v. into each recipient. Recipients were immunized with virus either on the day of transfer or had been immunized 6 days previously (see below).

Antiserum. Monoclonal anti-thy 1.2 was purchased from New England Nuclear Corp. (Boston, Mass.) and used at a final concentration of 1:5,000. Polyvalent rabbit anti-mouse immunoglobulin serum was prepared and tested as previously described (14, 16). Before use, this serum was heat inactivated at 56°C and then absorbed with normal mouse thymocytes and erythrocytes. For treatment with either antiserum, 10^8 lymph node cells per ml were suspended in diluted antiserum, incubated for 1 h on ice, and washed with balanced salt solution. The cells were then suspended in 1:6-diluted guinea pig serum (10^8 cells per ml) and incubated for 5 min on ice, followed by 40 min at 37°C. After being washed, the cells were suspended to 2×10^8/ml in balanced salt solution, and 0.5 ml was injected i.v. into each recipient.

Assay for endogenous proliferation. To examine proliferation in the draining lymph nodes in mice from various groups, lymph nodes were removed on 3 consecutive days after s.c. immunization with virus. The cells were adjusted to 4×10^6/ml in RPMI 1640 supplemented with 5% fetal calf serum, and 0.2 ml was placed in microtiter wells (Linbro round-bottom plates). Groups were tested in quadruplicate. One microcurie of [^{3}H]thymidine (New England Nuclear) was added to each well, and the cells were incubated for 4 h at 37°C under 5% CO_2. Wells were harvested with an automated cell harvester.

RESULTS

Induction and specificity of tolerance to HSV. Studies of DH to haptens have shown that the i.v. injection of either the soluble form of the hapten or haptenated cell membranes can induce a state of specific unresponsiveness (for a review, see reference 5). To determine whether the DH response to HSV could be similarly affected, mice were injected i.v. with 10^8 PFU of HSV or 50×10^6 HSV-SC 7 days before s.c.

immunization with the virus. The mice were ear challenged with 10^6 PFU of UV-inactivated HSV 6 days after immunization, and increased ear swelling was measured 24 h later. Control mice were immunized and challenged but did not receive the i.v. injection. The results of one such series of replicate experiments are given in Fig. 1. Experiment 1 shows that mice injected i.v. with HSV particles 7 days before immunization did not develop a significant DH response compared with immunized mice which were not pretreated with virus i.v. Further studies indicated that wild-type virus, as well as the temperature-sensitive mutant, could induce tolerance (data not shown).

This unresponsiveness was specific since mice injected i.v. with HSV particles were fully responsive to VSV, an unrelated virus. Reciprocally, mice could be made tolerant for DH to VSV by the i.v. injection of VSV 7 days before immunization with homologous virus. These mice showed normal DH responses to HSV (data not shown). However, in a more stringent test of specificity, mice were injected i.v. with HSV-1 particles and then immunized and challenged with HSV-2 (Fig. 1, experiment 2). The DH response of these mice to HSV-2 was significantly reduced. Thus, although tolerance for DH to HSV-1, induced by the i.v. injection of HSV-1 particles, is virus specific, it does not appear to by type specific.

Mice were injected with 50×10^6 HSV-SC 7 days before the s.c. immunization (Fig. 1, experiment 3). Upon challenge 6 days later, these mice gave significantly suppressed responses compared with controls. Again, this tolerance was antigen specific in that the injection of 50×10^6 HSV-SC had no effect on the response to the unrelated virus, VSV. Thus, the i.v. injection of either HSV particles or HSV-SC induced an antigen-specific state of unresponsiveness.

Demonstration and mode of action of suppressor cells induced by HSV particles and HSV-SC. To examine whether the mechanisms of toler-

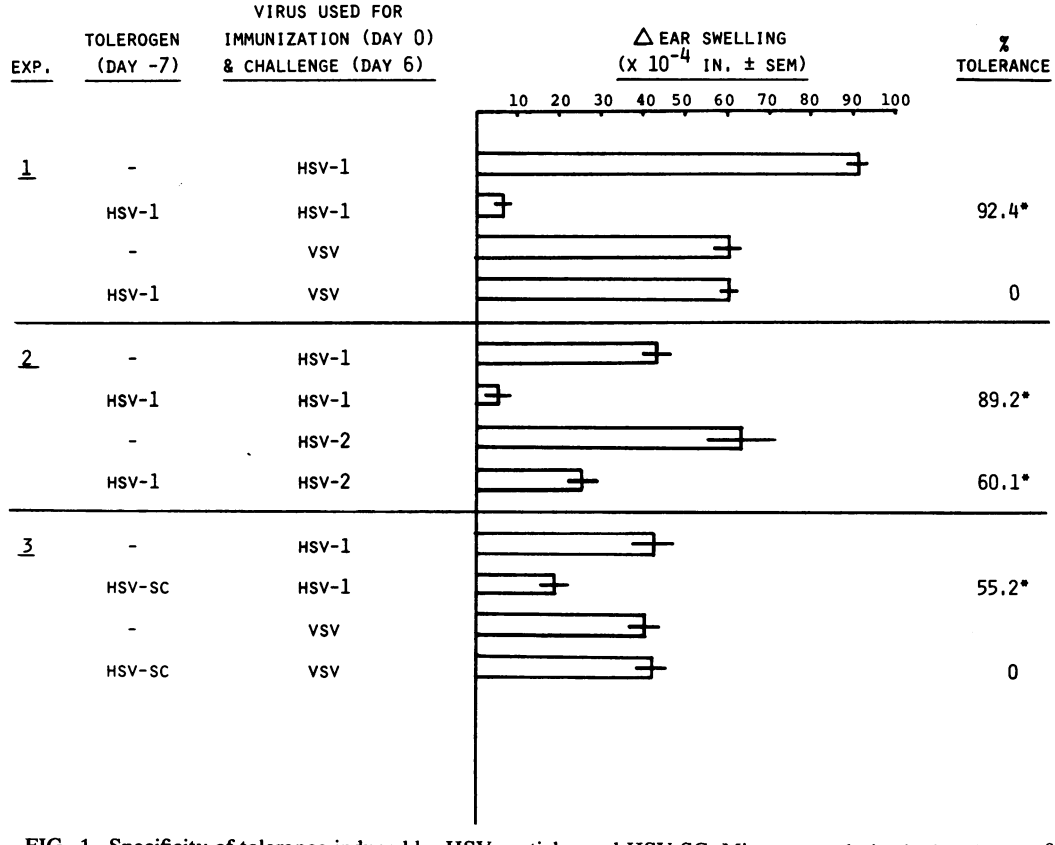

FIG. 1. Specificity of tolerance induced by HSV particles and HSV-SC. Mice were tolerized with either 10^8 PFU HSV or 50×10^6 HSV-SC, immunized with 4×10^6 PFU s.c. 7 days later, and challenged 6 days after immunization. The percent tolerance was calculated as described in Table 1, footnote *c*, and each group represents four mice. *, $P < 0.01$.

ance to HSV were similar for HSV particles and HSV-SC, we investigated the presence and characteristics of suppressor cells. Studies of tolerance to haptens have demonstrated that, depending on the form of the hapten and the tolerization regime, suppressor cells are induced which can act on different limbs of the DH response (2, 5, 13, 14). Thus, experiments were designed to determine whether suppressor cells were present and whether they affected the induction (afferent limb) or elicitation (efferent limb) of the DH response. Donor mice were tolerized either with HSV particles (Table 1, experiment 1) or HSV-SC (Table 1, experiment 2). Seven days later, 10^8 lymph nodes and spleen cells were transferred either to normal recipients that had been immunized the same day (afferent test) or to recipient mice that had been immunized 6 days previously (efferent test). Table 1, experiment 1, shows that cells derived from HSV-particle-tolerized mice could suppress DH when transferred on the day of immunization (group B) but not on the day of challenge (group C). In contrast, cells from mice tolerized with HSV-SC (Table 1, experiment 2) suppressed DH not only when transferred to normal recipients at the initiation of the response (group B) but also in preimmunized mice which received cells on the day of challenge (group C). These results indicate that the i.v. injection of HSV particles induces a suppressor cell which acts on the induction phase of the DH response, whereas HSV-SC induces suppressor cell(s) which either affect only the expression or both the induction and the expression of the DH response. Since it was possible that the suppression transferred by cells from HSV-SC-injected mice was mediated only by efferent acting cells, experiments were done to examine this population for afferent inhibition. In other systems, afferent-acting suppressor cells have been shown to inhibit proliferation in the draining lymph nodes of sensitized mice (14). Donor mice were tolerized with HSV-SC, and suppressor cells were transferred to recipient mice which were immunized the same day. Cells were taken from these recipient mice 4, 5, and 6 days later and tested in vitro for endogenous proliferation. Figure 2 shows that the endogenous proliferation of draining lymph node cells from mice given suppressors was significantly inhibited compared with that in immunized controls. The implication of these results is that the i.v. injection of HSV-SC induces two distinct types of suppressor cells, capable of inhibiting both the induction and the expression of DH to HSV.

Specificity of suppressor cells. To test the specificity of the suppressor cells induced by HSV particles and by HSV-SC, we examined these cells for their ability to inhibit the response to VSV. The DH response to VSV of mice receiving suppressor cells induced by HSV particles was not suppressed (Fig. 3, experiment 1). In other experiments, we found that the response to VSV could be inhibited by suppressor cells induced with VSV (data not shown). However, as seen with tolerance in the donor mice, suppressor cells induced by HSV-1 particles did have a significantly suppressive effect on the DH response to HSV-2 (Fig. 3, experiment 2). Both types of suppressor cells induced by HSV-SC were found to act in an antigen-specific manner (Table 2). The transfer of 10^8 suppressor cells from donors given HSV-SC had no effect on DH to VSV when given either on the day of immunization (Table 2, experiment 1) or on the day of challenge (Table 2, experiment 2).

Characterization of suppressor cells. To investigate the characteristics of the suppressor cells induced by the i.v. injection of HSV particles and HSV-SC, we treated spleen and lymph node cells from tolerized mice with anti-Thy 1.2, rabbit anti-mouse immunoglobulin serum, or normal rabbit serum and complement and as-

TABLE 1. Mode of action of suppressor cells induced by HSV particles and HSV-SC

Expt	Group	Tolerogen[a]	Transfer from HSV-1-tolerant donors	Day of transfer[b]	Δ Ear swelling (10^{-4} in. ± SEM)	% Suppression[c]
1	A	—[d]	—		54.8 ± 3.7	
	B	HSV particles	10^8 spleen + lymph node cells	0	16.5 ± 2.0	70.1[e]
	C	HSV particles	10^8 spleen + lymph node cells	6	58.0 ± 6.1	
2	A	—	—		36.5 ± 5.5	
	B	HSV-SC	10^8 spleen + lymph node cells	0	10.1 ± 4.3	72[e]
	C	HSV-SC	10^8 spleen + lymph node cells	6	0.6 ± 5.1	99[e]

[a] Suppressor cells were obtained by the i.v. injection of HSV particles or infected cells 7 days before transfer.
[b] Cells were transferred to recipients either on the day of immunization (group B) or 6 days later, on the day of challenge (group C).
[c] The percent suppression was calculated as follows: [1 − (Δ tolerized/Δ immunized)] × 100.
[d] —, None.
[e] $P < 0.01$.

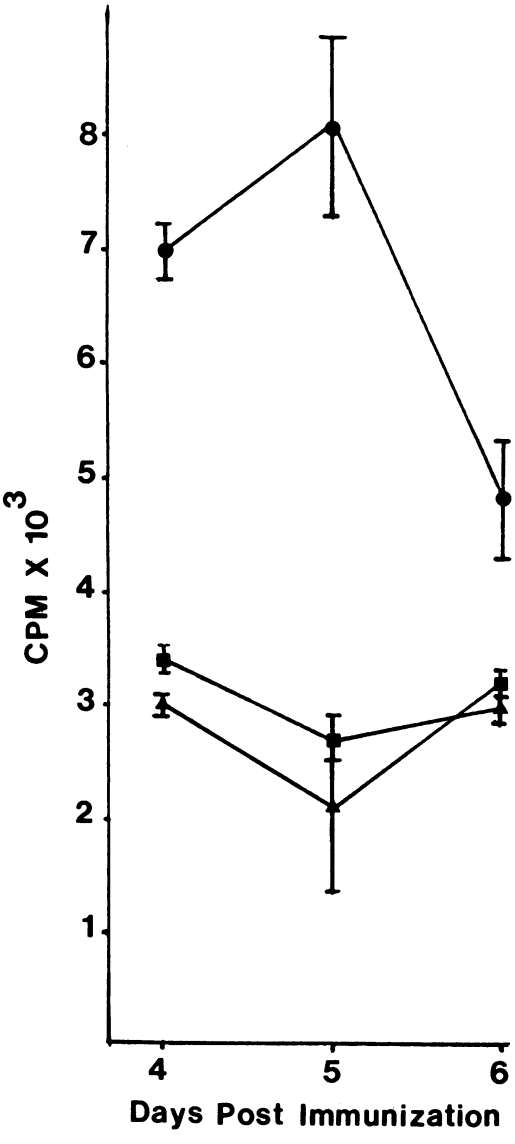

FIG. 2. Endogenous proliferation in lymph nodes of afferent suppressed mice. Mice received 100 × 10^6 lymph nodes plus spleen cells from donors tolerized with 50 × 10^6 HSV-SC 7 days before transfer. Recipients and normal mice (▲) were immunized with 4 × 10^6 PFU s.c. On days 4 and 5 and after the day of transfer and immunization, lymph node cells from recipient mice (■), immunized mice (●), and naive mice were tested for proliferation in vitro during a 4-h period. Each point is the mean of four values and groups of two mice.

sayed them for their ability to transfer suppression. Figure 4 shows that for each suppressor cell population, treatment with normal rabbit serum or rabbit anti-mouse immunoglobulin serum had no effect on the transfer of the suppression of DH to HSV. However, suppressor populations treated with anti-Thy 1.2 lost suppressor activity. The transfer of serum from HSV-1-tolerized mice did not cause significant suppression. Together, these findings indicate that the suppressor cells induced by the i.v. injection of HSV particles and HSV-SC are T cells (T_s).

DISCUSSION

In the series of experiments presented here, we have shown that tolerance for DH to HSV-1 can be induced by the i.v. injection of either HSV particles or HSV-SC 7 days before immunization. With either form of tolerogen, the state of unresponsiveness is virus specific, i.e., mice tolerant to HSV-1 give normal responses to VSV, but not type specific since HSV-1-tolerant mice are significantly unresponsive to HSV-2 as well (data only shown for HSV particles). Tolerance to HSV induced by HSV particles or HSV-SC is at least partially mediated by T_s cells, which can be demonstrated by transfer and characterized by their mode of action. T_s cells induced by HSV particles appear to block only the afferent limb of the immune response and have no effect when given to previously sensitized mice. However, the i.v. injection of HSV-SC induces two suppressor activities: one which blocks proliferation at the initiation of the response and a second which blocks the efferent stage of DH to HSV.

The i.v. injection of either HSV-SC or HSV particles appears to induce a state of split tolerance. Mice tolerized for DH develop a neutralizing antibody titer of 1:64 to 1:128 within 7 days, which rises to 1:128 to 1:256 by 14 days. In contrast, s.c. immunization to induce DH does not produce detectable antibody by day 6 (as measured by 4-h ear swelling, immunoprecipitation, or neutralization) and only low levels by day 14 (neutralizing titer of 1:32 to 1:64). Since the development of neutralizing antibody to HSV is T cell dependent (4), the i.v. injection of HSV appears to selectively affect DH. This is supported by a report which indicates that mice tolerant for DH do contain cytotoxic T cells to HSV (23).

Although the i.v. induction of T_s cells has been described for many soluble and membrane-bound antigens, no definitive pattern has emerged in terms of which type of tolerogen will induce which population(s) of suppressor cells. The HSV particle preparation which induces tolerance and the afferent-acting T_s cells contains viral particles, soluble viral proteins, and fragments of infected rabbit skin cell membranes. The HSV-SC preparation probably contains some soluble proteins and particles, as well as the infected syngeneic cells. It should be

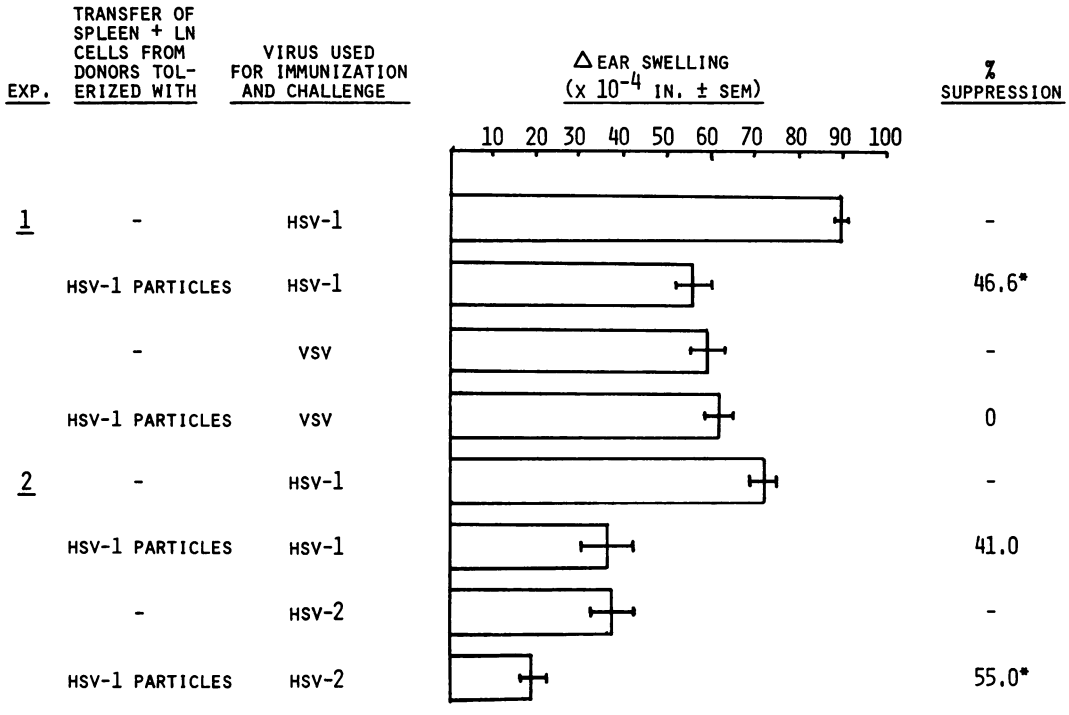

FIG. 3. Transfer of specific suppression of DH with spleen and lymph node (LN) cells from HSV-tolerant donors. Donors were tolerized with 10^8 PFU of HSV particles 7 days before s.c. immunization with 4×10^6 PFU. Mice were ear challenged 6 days after immunization, and the percent suppression was calculated as described in Table 1, footnote *c*. Each group represents data from four mice. *, $P < 0.01$.

noted that at 4 h after the infection of spleen cells with HSV, membrane-associated viral glycoproteins could not be detected by immunofluorescence staining with rabbit anti-HSV antibody and fluorescenated goat anti-rabbit immunoglobulin. However, by 8 to 12 h postinfection, 85% of the cells show positive membrane staining, indicating that the viral proteins are probably integrated into the plasma membrane and not simply adhering to the surface of the spleen cell. Collectively, these observations suggest that the afferent-acting T_s cells may be induced directly by HSV particles or soluble proteins or both, whereas the efferent-acting T_s cells may be dependent upon the recognition of viral proteins in association with self antigens.

TABLE 2. Specificity of suppressor cells induced by HSV-SC

Expt	Recipient mice injected with 10^8 cells from HSV-SC-tolerized donors[a]	Day of transfer to immunized mice[b]	Virus used for immunization (day 0) and challenge (day 6)	Δ Ear swelling (10^{-4} in. ± SEM)	% Suppression[c]
1	−		HSV	82.3 ± 8.8	
	+	0	HSV	39.3 ± 3.7	53.3[d]
	−		VSV	47.8 ± 5.5	
	+	0	VSV	46.2 ± 5.3	3.3
2	−		HSV	64.9 ± 7.9	
	+	6	HSV	30.5 ± 4.0	46.0[d]
	−		VSV	72.4 ± 3.2	
	+	6	VSV	75.8 ± 3.8	0

[a] Donors were tolerized with 50×10^6 HSV-SC 7 days before transfer.
[b] Recipients were either immunized (4×10^6 PFU s.c.) the same day that they received suppressor cells (day 0) or had been immunized 6 days previously.
[c] The percent suppression was calculated as described in Table 1, footnote *c*.
[d] $P < 0.01$.

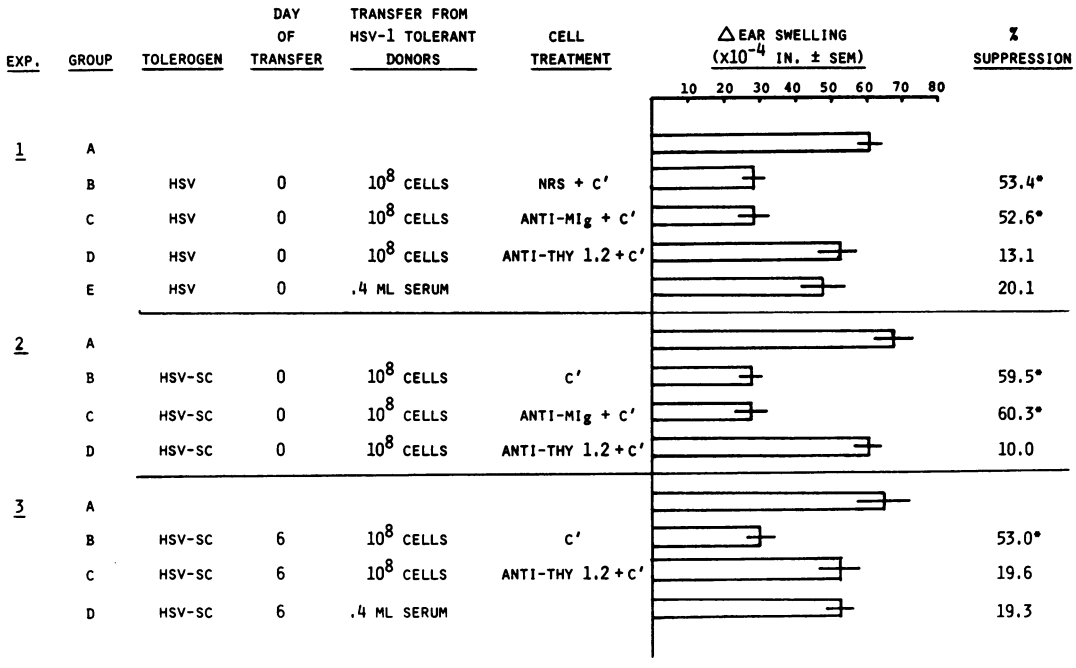

FIG. 4. Phenotype of cells which transfer suppression. Suppressor cell donors were tolerized with either 10^8 HSV particles or 50×10^6 HSV-SC 7 days before transfer. Cells were treated with antisera or complement or both and transferred to recipient mice which were immunized (4×10^6 PFU s.c.) the same day (day 0) or which had been immunized 6 days previously. In experiments 1 and 2, mice were challenged 6 days after the transfer of cells or serum. In experiment 3, mice were challenged the same day that they received treated cells or serum. Each group represents four mice, and the percent suppression was calculated as described in Table 1, footnote *c*. NRS, Normal rabbit serum; C', complement; and anti-MIg, rabbit anti-mouse immunoglobulin serum.

Which particular infected cell(s) in the HSV-SC population is required for the induction of the T_s cells has not yet been determined.

Tolerance for DH induced by i.v. injection has been extensively studied with haptens (5), and investigators have speculated that these systems may closely reflect the regulation of anti-viral responses in that a hapten-derivitized protein in a host cell membrane and a viral protein in a cell membrane may be recognized in a similar manner. Recently, several groups have begun to investigate the regulation induced by the i.v. injection of viruses. With respect to HSV, it has been shown by other investigators that the i.v. injection of an infected cell lysate resembling our HSV particle preparation induces tolerance and suppressor cells for DH to HSV, although the T_s cells are not detectable until 14 days after i.v. injection (20, 21). In other systems, the characteristics of the unresponsive state and the types of suppressor cells induced vary with the virus used and its infectivity. For example, in studies with reovirus, Greene and Weiner (6) found that mice injected with inactivated virus were tolerant for DH, and T_s cells were demonstrated. In contrast to our finding that either infectious or temperature-sensitive HSV can induce tolerance, the i.v. injection of infectious reovirus primed the mice for DH. Thus, with reovirus, the ability of the virus to infect the host appears to circumvent the induction of tolerance. It should be noted, however, that the i.v. injection of inactivated virus does not always induce tolerance for DH. In studies with influenza, Leung et al. (10) found that the i.v. injection of UV-inactivated influenza virus induced DH but tolerized cytotoxic T cells. T_s cells were present in the tolerant mice which inhibited the development of specific cytotoxicity upon transfer. These differences in immune regulation for different viruses probably relate to the natural routes taken during pathogenesis and the differential necessity for the positive and negative control of each response.

The specificity of tolerance and suppressor cells to viruses is complex and not yet well understood. For unrelated viruses, tolerance appears to be specific, but among related viruses, the findings vary. In agreement with our results, Nash et al. (20) found that the i.v. injection of HSV-1 caused a significant reduction in the response to HSV-2. This cross-

reactivity probably reflects the tolerization of the DH response to type-common determinants while leaving responses to the type-specific antigenic determinants intact. In the reovirus system, tolerance and suppressor cells appear to be serotype specific (6). As mentioned above, the i.v. injection of influenza virus did not tolerize DH. However, aerosol inoculation did induce tolerance and T_s cells for DH, and these T_s cells were specific for the homologous viral hemagglutinin but could not distinguish between serotypically different strains (11).

Currently, the biological significance of tolerance for DH induced by the i.v. injection of HSV is unknown. However, since HSV viremias occur in neonates and immunosuppressed patients (3, 8, 19), the induction of hyporesponsiveness by this method may occur in vivo, contributing significantly to the dissemination of the infection. Whether circulating infected lymphocytes are present in either acutely or latently infected individuals has not been determined, although this is not an unlikely possibility. It is also possible that this type of immune regulation may have a positive role in HSV infection in that it may prevent excess DH and minimize immunopathology. For instance, evidence exists that T cells can increase pathology in a mouse keratitis model (12). Also, since HSV resides in nervous tissue, one might speculate that careful regulation of a local inflammatory response would be beneficial. Eventually, the elucidation of the positive and negative roles of regulatory and immune cells may have important implications for treatment in both acute and latent HSV infections.

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