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Antigen-Specific Immune-Suppressor Factor in Herpes Simplex Virus Type 2 Infections of UV B-Irradiated Mice

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UV B-irradiation (280 to 320 nm) of mice at the site of cutaneous infection with herpes simplex virus type 2 (HSV-2) induced suppressor T-cell circuits that decreased HSV-2-induced proliferative responses of HSV-2-immune lymph node cells. Adoptive transfer experiments indicated that splenocytes from UV B-irradiated HSV-2-infected animals contain L3T4$^+$ cells that suppress proliferative responses in vivo, consistent with suppressor inducer cells. However, following in vitro culture of the splenocytes with HSV-2 antigen, the proliferation of immune lymph node cells was inhibited by Lyt2$^+$ suppressor T cells, consistent with antigen-induced suppressor effector cells. Antigen-specific and nonspecific suppressor factors were fractionated from supernatants of HSV-2-stimulated spleen cells by molecular-sieve chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Sephadex fraction that contained the antigen-specific suppressor factor, in the presence or absence of 2-mercaptoethanol, defined a 115-kilodalton protein consisting of two disulfide-bound components with molecular sizes of 70 and 52 kilodaltons. The implications of these results with respect to the regulation of HSV-induced cell-mediated immunity following UV B-irradiation are discussed.

Immune responsiveness to noninfectious antigens is regulated by suppressor T (Ts)-cell circuits composed of various T-cell subsets and soluble antigen-specific and nonspecific factors (1, 2, 7) that mediate the ultimate suppressor effect (14). Similar pathways have also been described for infectious agents (4, 9, 15), but their role, if any, in the disease process remains unclear. Recently, we showed that recrudescent disease due to herpes simplex virus (HSV) is associated with a significant increase in the proportion of Ts cells and the elaboration of soluble suppressor factors (SFs) (13, 16–18). We proposed that, by inhibiting protective immunity, Ts-cell circuits play an important role in recrudescent disease (6, 13, 18). However, further verification of this hypothesis requires a better understanding of Ts-cell subsets and SFs that are generated by recrudescence-inducing stimuli.

UV B-irradiation, an established recrudescence-inducing stimulus (3, 10), causes the suppression of HSV-induced delayed-type hypersensitivity responses and increases the severity of HSV-induced cutaneous disease (20, 21). This may involve Ia$^+$ epidermal cells, since Ia$^+$ epidermal cells have antigen-presenting function in HSV type 2 (HSV-2)-induced T-cell proliferation (LT), a function that is impaired by in vitro irradiation of the epidermal cells (11). In this report, we discuss the nature of the Ts cells and the SF(s) that regulate HSV-2-induced LT responses in UV B-irradiated mice and describe an antigen-specific SF that may be involved in the generation of the ultimate suppressor effect.

Female BALB/c (*H-2^d*) mice (Jackson Laboratory, Bar Harbor, Maine), 8 to 12 weeks old, were irradiated (four consecutive days; 33.4 mJ/cm^2 per day) on the shaved chest walls and infected with HSV-2 by intradermal inoculation (6 $\times$ 10^5 PFU) at the site of irradiation (21). Sixty percent of the energy emitted by the FS20 sunlamps (Westinghouse, Bloomfield, N.J.) used in these series is in the UV B region (280 to 320 nm), with a peak at 313 nm (8). Spleen cells (SC) or draining lymph node cells (LNC) were obtained at 8 days postinfection. The cells were cultured (4 days, 37°C) with HSV-2 antigen (20 μg of protein per ml) prepared as described elsewhere (6, 11, 21). Proliferative responses were measured in a 6-h pulse assay with 1 μCi of tritiated thymidine (^{3}H-TdR; New England Nuclear Corp., Boston, Mass.) per well. The data are expressed as net counts per minute calculated as follows: net counts per minute = mean counts per minute experimental − mean counts per minute medium control (11, 21).

LNC from HSV-2-infected mice (8 days postinfection) were used as immune responder cells in mixed cell cultures. The cells were mixed with various proportions of regulatory cells consisting of SC from HSV-2-immunized UV B-irradiated mice that had been treated with mitomycin C (50 μg/ml; 30 min, 37°C) prior to mixing. The resulting combinations contained 100, 80, 50, 20, and 0% immune responder cells with 0, 20, 50, 80, and 100% regulatory cells, respectively. These mixed cultures (4 $\times$ 10^5 cells per well) were grown (4 days, 37°C) with HSV-2 antigen (20 μg of protein per ml) and assayed for ^{3}H-TdR incorporation as described elsewhere (13). In some experiments, regulatory cells consisted of SC depleted of specific T-cell subsets by complement-mediated lysis with monoclonal anti-L3T4 (obtained from G. Cole, Baltimore, Md.) or anti-Lyt2.2 (New England Nuclear) antibody. Briefly, cells were incubated (30 min, 4°C) with the respective monoclonal antibodies followed by complement (rabbit low tox; Accurate Scientific Chemical Co., Westbury, N.Y.) (30 min, 37°C) (11, 21). The effectiveness of depletion was confirmed by the failure of the remaining cells to stain with the respective monoclonal antibodies in immunofluorescence assays performed as described earlier (18).

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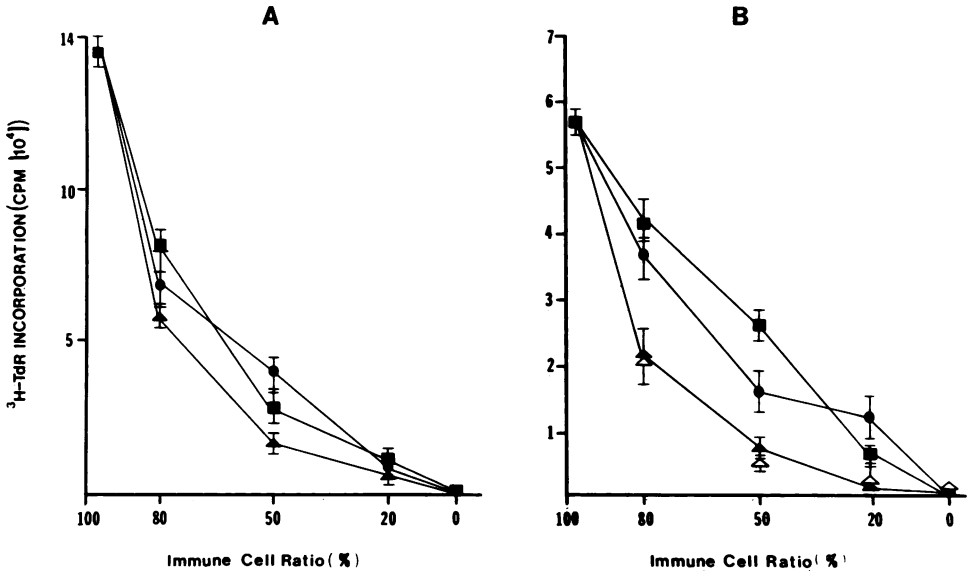

FIG. 1. ^{3}H-TdR incorporation in mixed cell cultures. LNC from HSV-2-immune animals (immune responder cells) were mixed with various proportions (100, 80, 50, 20, and 0%) of regulatory cells not precultured (A) or precultured (B) with HSV-2 antigen (20 μg of protein per ml; 4 days, 37°C). Both regulatory cell preparations were treated with mitomycin C (50 μg/ml; 30 min, 37°C) prior to mixing. The regulatory cells were SC from HSV-2-infected UV B-irradiated mice (▲), from HSV-2-infected nonirradiated mice (●), or from HSV-2-infected UV B-irradiated mice precultured with HSV-2 antigen and depleted of L3T4$^+$ cells by complement-mediated lysis (△). Mitomycin C-treated SC from nonimmune mice (■) served as dilution control. Mixed cultures were incubated with HSV-2 antigen (20 μg of protein per ml) for 4 days and assayed for ^{3}H-TdR incorporation. Results are expressed as mean counts per minute ± standard deviation.

For adoptive transfer, normal recipients were injected intravenously via the tail vein (1×10^8 cells per mouse in 0.2 ml of RPMI 1640) (20, 21). One hour after transfer, recipients were inoculated (intradermally in the chest) with 6×10^5 PFU of HSV-2.

SF activity was assayed as described earlier (11, 13, 21), on the basis of the ability of lymphoid culture supernatants to decrease the LT responses of HSV-2- (HSV specific) or vaccinia (VTK$^-$79) virus- (nonspecific) immune LNC when added at a 20% final concentration at the time of culture initiation. SF activity, expressed as the percentage of suppression, was calculated from the following formula: percent suppression = (1 − [mean counts per minute experimental/mean counts per minute control]) × 100, where mean counts per minute experimental is the mean counts per minute for RPMI 1640 with 10% horse serum and culture supernatant and mean counts per minute control is the mean counts per minute for RPMI with 10% horse serum and viral antigen. For further SF characterization, lymphoid culture supernatants were fractionated by Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) chromatography and fractions were assayed for the ability to decrease HSV-2- or VTK$^-$79-induced LT responses (11, 13, 21). Fractions containing HSV-specific activity were pooled and precipitated with 5% trichloroacetic acid, denatured (20 μl of 10% NaOH; 30 μl of distilled water; 20 μl of denaturing solution consisting of Tris hydrochloride [pH 7.0], 2% sodium dodecyl sulfate, 0.005% bromothymol blue, and 6% sucrose with [reducing] or without [nonreducing] 5% 2-mercaptoethanol [2-ME]), and electrophoresed on sodium dodecyl sulfate–8.5% polyacrylamide gels. The gels were stained with a silver stain as described elsewhere (17).

The proliferative responses of mixed cell cultures are shown in Fig. 1. The magnitude of ^{3}H-TdR incorporation in mixed cultures containing mitomycin C-treated regulatory cells (SC from UV B-irradiated mice) represented a virtually linear dilution of the immune responder cells. The response was identical to that observed in mixed cultures in which the regulatory cells were similarly treated nonimmune SC or SC from HSV-2-immune nonirradiated mice (Fig. 1A). However, SC from UV B-irradiated HSV-2-immune animals that were first cultured with HSV-2 antigen (20 μg of protein per ml; 4 days), treated with mitomycin C, and then mixed with immune LNC showed a significant ($P < 0.01$) reduction in the levels of ^{3}H-TdR incorporation, even at a 20% cell concentration (Fig. 1B). A similar reduction in the magnitude of ^{3}H-TdR incorporation was observed when the antigen-precultured SC from irradiated mice were depleted of L3T4$^+$ cells prior to mixing (Fig. 1B). Some reduction in ^{3}H-TdR incorporation was also observed in cultures containing SC from nonirradiated animals that had been precultured with HSV-2 antigen. However, the reduction was significantly lower and was detectable only when the regulatory cells were mixed in equal proportions with immune responder cells (immune cell ratio, 50%) (Fig. 1B). We interpret these data to indicate that following in vitro stimulation with HSV-2 antigen, splenocytes from UV B-irradiated HSV-2-infected mice contain a Lyt2$^+$ T-cell subset capable of reducing the LT responses of HSV-2-immune LNC.

Previous depletion studies (with anti-Thy1.2 antibody) have shown that suppression is mediated by T cells (21). To further identify these Ts cells, SC from UV B-irradiated HSV-2-infected mice were depleted of various cell subpopulations by complement-mediated lysis with anti-L3T4 or anti-Lyt2.2 antibody and transferred to naive recipients. The recipients were infected with HSV-2 within 1 h after cell transfer, and their LNC (obtained at 8 days postinfection) were assayed for HSV-2-induced proliferative responses. ^{3}H-TdR incorporation was significantly reduced ($P < 0.01$) in cultures of LNC from the recipients of nondepleted and Lyt2$^+$-depleted SC. Adoptive transfer of L3T4$^+$-depleted SC from UV B-irradiated animals failed to suppress (Fig. 2).

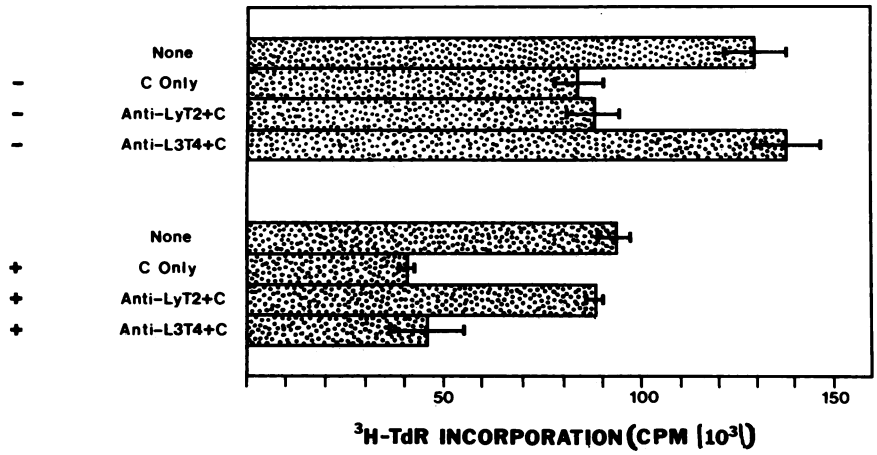

FIG. 2. Adoptive transfer of UV B-irradiation-induced suppressor cells. SC from UV B-irradiated mice precultured (+) with HSV-2 antigen (20 μg protein per ml; 4 days, 37°C) or not (−) were depleted of various T-cell subsets by complement (C)-mediated lysis and transferred to naive recipients (1×10^8 cells per mouse). The recipients were infected with HSV-2 (6×10^5 PFU) within 1 h after cell transfer. LNC obtained 8 days later were grown with HSV-2 antigen (20 μg of protein per ml) for 4 days and assayed for ^{3}H-TdR incorporation. Results are expressed as mean counts per minute ± standard error.

In contrast, when the SC from UV B-irradiated mice were first precultured with HSV-2 antigen and then depleted of various T-cell subsets, only the recipients of nondepleted or L3T4$^+$-depleted SC evidenced reduced LT responses (Fig. 2). There was no decrease in the LT responses of recipients of SC from nonirradiated HSV-2-infected mice. We interpret these data to indicate that splenocytes from UV B-irradiated HSV-2-infected mice contain an L3T4$^+$ cell subset that can reduce the LT responses of immune LNC following adoptive transfer and in vivo exposure to HSV-2 antigen (consistent with suppressor inducer cells). However, as in mixed cell cultures, the Ts cells detected following in vitro stimulation of SC with HSV-2 antigen were Lyt2$^+$.

Supernatants of HSV-2-stimulated SC from UV B-irradiated mice suppressed HSV-2-induced LT responses of immune LNC (73% suppression). Consistent with our previous report (21), supernatants of similarly cultured SC from nonirradiated mice had only minimal suppressive potential (9% suppression). We think that the SF activity was not directly mediated by the viral proteins in the SC cultures because (i) it was virtually absent from similarly cultured SC from nonirradiated mice and (ii) the nonsuppressive control medium was artificially supplemented with viral antigen. To further characterize these suppressor activities and to determine their antigenic specificity, supernatants were fractionated on Sephadex G-100 columns and the fractions were assayed for the ability to inhibit HSV-2- or VTK$^-$79-induced LT responses. The factor(s) that inhibited HSV-2-induced LT responses of HSV-2-immune LNC was localized within two fractions with approximate molecular sizes of 20 to 40 and 90 to 120 kilodaltons (kDa) (Fig. 3). The lower-molecular-size fraction (20 to 40 kDa) also inhibited VTK$^-$79-induced proliferation. However, the SF activity of the higher-molecular-size fraction was HSV specific and was designated HSV-SF. Only one fraction (molecular size, 25 to 20 kDa) had SF activity in similarly fractionated supernatants of SC from nonirradiated mice, and it inhibited both HSV-2- and VTK$^-$79-induced LT responses. This activity did not exceed 5 ± 3% suppression.

Sephadex fractions containing the HSV-SF activity from SC of UV B-irradiated mice and the corresponding HSV-SF-negative fractions from supernatants of SC from nonirradiated mice (Fig. 3) were independently pooled, trichloroacetic acid precipitated, and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing (with 2-ME) and nonreducing (without 2-ME) conditions. Two bands with molecular sizes of 70 and 52 kDa were observed in the HSV-SF-positive fractions under reducing conditions (Fig. 4A, lane 2). These bands were replaced by one 115-kDa band when electrophoresis was done under nonreducing conditions (Fig. 4B, lane 1). These bands were not observed in the HSV-SF-negative fractions (Fig. 4A, lanes 1 and 3 and Fig. 4B, lane 2) from supernatants of nonirradiated SC or from other fractions of the column (Fig. 3). A third, 67-kDa band that comigrated with bovine serum albumin (Fig. 4, lane 3) was detected in both the HSV-SF-positive and -negative fractions, and it probably represents a serum contaminant from the culture medium.

We interpret these data to suggest that HSV-SF is a dimer (molecular size, 115 kDa) that consists of two disulfide-bound proteins with molecular sizes of 70 and 52 kDa. This finding is consistent with previous results from hapten systems, in which antigen-specific SFs that play an important role in the transmission of suppressogenic signals were shown to be frequently composed of two subunits held together by disulfide bonds (1). However, unlike those SFs which sometimes bear antiidiotypic determinants (19), HSV-SF did not react in Western blot (immunoblot) assays (5) with a guinea pig anti-HSV-2 serum that shares idiotypes with a murine anti-HSV serum (data not shown). Different results were obtained by Horohov et al. (12), who described an HSV-specific SF in supernatants from HSV-1-stimulated splenocytes and showed that it consists of a 68.5-kDa subunit that bears antiidiotypic determinants. The exact reason for this difference is not clear. Technical interpretations include our use of an inappropriate antiserum for the detection of antiidiotypic determinants and the failure of Horohov et al. (12) to identify subunits other than the 68.5-kDa subunit (which may be equivalent to our 70-kDa band). Indeed, this latter interpretation is consistent with the conclusions of Horohov et al. (12). Nevertheless, we cannot exclude the possibility that the Ts-cell circuits induced by UV B-irradiation differ from those observed under normal antigen stimulation of immune cells, since HSV-2-stimulated

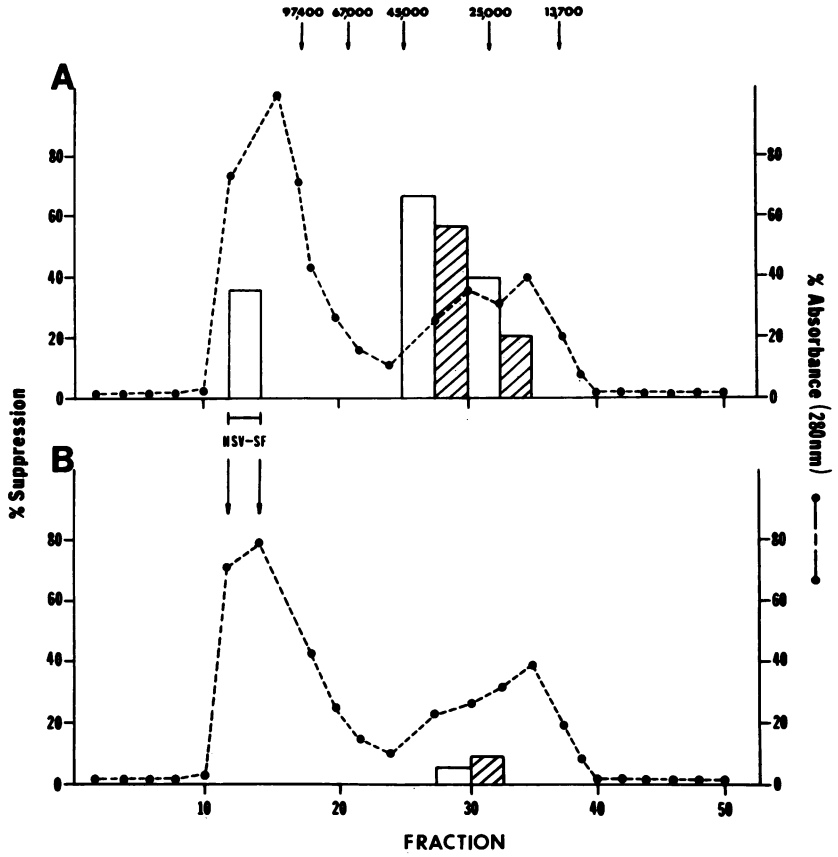

FIG. 3. Molecular-sieve chromatography of SFs. Supernatants from HSV-2-stimulated 4-day cultures of SC from UV B-irradiated (A) or nonirradiated (B) HSV-2-immunized mice were concentrated by lyophilization (1:10) and fractionated on a Sephadex G-100 column (0.7 by 20 cm) prepared in phosphate-buffered saline. Pools of four fractions (0.3 ml each; indicated by arrows in panel B) were assayed for SF activity (20% final concentration) on HSV-2- (□) and VTK⁻79- (▨) immune LNC. The molecular sizes and elution positions (arrows) of markers are indicated above panel A.

SC from nonirradiated mice have some suppressive potential (Fig. 1B) and the supernatants contain nonspecific SF activity (Fig. 3B).

At this time, we can only speculate on the role played by HSV-SF in the UV B-irradiation-induced immunosuppression associated with the increased severity of HSV disease (20, 21). By analogy with hapten systems, we assume that HSV-SF is part of the regulatory Ts-cell circuit that modulates HSV-induced immunity. HSV-SF may be the product of the UV B-irradiation-induced L3T4$^+$ splenocytes, since their suppressive activity is antigen specific (21). However, L3T4$^+$ cells (and presumably HSV-SF) failed to mediate suppression directly, as evidenced by the failure of SC from UV B-irradiated mice to reduce the proliferative responses of immune LNC (Fig. 1A) (suppressor inducer cells). Instead, recognition by another antigen-activated Lyt2$^+$ suppressor cell was required (Fig. 1B; 2). The suppressive activity of the Lyt2 cells appears to be non-antigen specific, at least as measured by delayed-type hypersensitivity re-

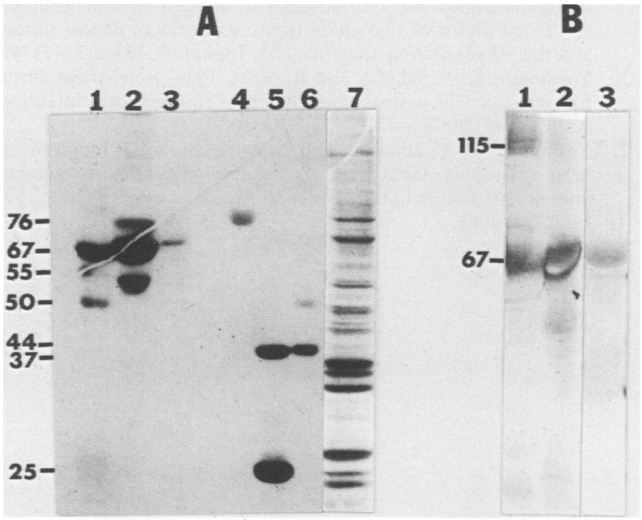

FIG. 4. Silver stain of sodium dodecyl sulfate–8.5% polyacrylamide gel. (A) Electrophoresis under reducing (with 2-ME) conditions. Lane 1, HSV-SF-negative fractions (fractions 11 to 14) from Sephadex column of SC from HSV-2-infected nonirradiated mice (Fig. 3B); lane 2, HSV-SF-positive fractions (fractions 11 to 14) from Sephadex column of SC from UV B-irradiated HSV-2-infected mice (Fig. 3A); lane 3, suppressor activity-negative fraction 22 from Sephadex column (Fig. 3A); lanes 4 to 6, molecular size markers including immunoglobulin (76 kDa), alcohol dehydrogenase (37 kDa), chymotrypsin (25.7 kDa), and ovalbumin (44 kDa); lane 7, HSV-2 antigen. (B) Electrophoresis under nonreducing (without 2-ME) conditions. Lane 1, HSV-SF-positive fractions shown in panel A, lane 2; lane 2, HSV-SF-negative fractions shown in panel A, lane 1; lane 3, bovine serum albumin.

sponsiveness (21). Presumably, HSV-SF is involved in the induction of this latter cell subset (suppressor effector cells). The $Lyt2^+$ suppressor effector cells respond by producing nonspecific suppressor factors that apparently act as the ultimate suppressors (14). Final conclusions must await the results of ongoing studies designed to further identify the interaction between HSV-SF, the nonspecific SF, and the various Ts-cell subpopulations defined in these studies.

It is tempting to consider these findings within the context of our previously stated hypothesis (6, 13, 18) that posits regulatory aspects of virus-specific immunity at the center of the recurrent disease problem. According to this hypothesis, UV B-irradiation would produce systemic, as well as local, changes in the skin (EC) that lead to a depression of defense mechanisms, including the generation of Ts cells. Possibly, Ts-cell circuits generated during UV B-irradiation are further activated on secondary exposure to the virus (either by reactivation of latent HSV or by exogenous reinfection with another HSV), thereby facilitating the development of clinically overt recurrent lesions despite the presence of immune memory. Indeed, both humans (16–18) and guinea pigs (6, 13) experience recurrent HSV-2 lesions associated with an increased proportion of Ts cells that down regulate virus-specific cell-mediated immune responses, and UV B-irradiation causes virus reactivation and recurrent disease (3, 10). If Ts cells and their products are indeed involved in modulating HSV immunity in general and during exposure to sunlight (UV B-irradiation) in particular, they could provide a useful target for breaking the recurrent disease cycle.

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