Regulation of Herpes Simplex Virus-Specific Lymphoproliferation by Suppressor Cells

DAVID W. HOROHOV,* ROBERT N. MOORE, AND BARRY T. ROUSE

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845

We investigated the regulation of the herpes simplex virus (HSV)-specific lymphoproliferative response (LPR) by suppressor cells. The chief cell types in HSV-immune splenocytes proliferating in response to the antigen were Lyt 1$^+$ and Lyt 2$^+$ T cells, which accounted for approximately 60 and 40% of the response, respectively. Because the total responsiveness of splenocytes was enhanced after depletion of Lyt 2$^+$ cells, the LPR was assumed to be subject to regulation by an Lyt 2$^+$ suppressor cell. This was shown to be the case with an experimental design in which suppressor cell activity was induced in one culture, the cells were irradiated, and the effects on LPR were measured in a test antigen-stimulated culture. The cell responsible for suppression was shown to be Lyt 2$^+$ IJ$^+$, and the actual suppressor effect was not antigen specific. Cellular requirements for the generation of suppression were also investigated. The three distinct cell types that appeared to be required were Lyt 2$^+$ and Lyt 1$^+$ T cells and an IJ$^+$ antigen-presenting cell. Of the three cell types, only the Lyt 2$^+$ cell needed to be from HSV-immune animals. The implications of our model system for the better understanding of the role of immunity in herpesvirus pathogenesis are discussed.

A variety of research approaches have indicated that cellular aspects of immunity play a principal role in recovery from herpesvirus infections (8, 13). However, herpesviruses persist in the body and may cause recrudescent disease despite the presence of effector T cells and antibody. No satisfactory explanation exists to explain why recrudescent lesions occur and why their severity varies. However, one idea currently courting favor is that changes in immunoregulation resulting from suppressor cell activity precede and account for lesion development (15, 16). Support for this idea includes the observation that changes in the ratio of suppressor to helper cells may occur around the time of recrudescence (15). As a prelude to studying the regulation of immunity in animal models of recrudescence, we approached the question of the role that suppressor mechanisms might play in influencing T cell immunity as measured by the lymphoproliferative response (LPR) assay. We demonstrated the contribution of different T cell subsets to the LPR and the extent of LPR regulation by suppressor cells. Finally, we defined some of the cell types involved in suppressor cell induction.

MATERIALS AND METHODS

Virus preparations. Herpes simplex virus (HSV) type 1 strain KOS was propagated in HEp-2 cells as described before (11). The viral stock had an infectivity titer of 4 × 10^8 PFU/ml. UV-inactivated HSV was prepared by exposing 0.5 ml of the viral stock to a germicidal lamp (Sylvania Electric Products, Danver, Mass.) at a distance of 3 cm for 2 min. This resulted in a reduction of the viral titers to fewer than 10^2 PFU/ml. Heat inactivation was performed by incubating 0.5 ml of the viral stock at 60°C for 30 min. The infectivity titer of heat-inactivated HSV stock was less than 10 PFU/ml. Sonicated HEp-2 cells were used as sham controls in all experiments. Influenza virus strain A/PR8/34 was propagated in embryonated chicken eggs. Influenza with stocks contained 1,200 hemagglutination units per ml.

Mouse immunization and splenocyte cultures. C3H/HeJ

mice (4 to 6 weeks old) were obtained from the breeding colony of the University of Tennessee Memorial Research Center Hospital, Knoxville, Tenn. The mice received a single injection of 10^6 PFU of HSV in a 0.1-ml volume by various routes. HSV-immune mice received an intraperitoneal injection 4 weeks before use. Influenza-immune mice received an intraperitoneal injection of 40 hemagglutination units 4 weeks before use.

The preparation of single-cell suspensions of splenocytes has been described elsewhere (11). The splenocytes were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 5% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and 5 × 10^5 mM 2-mercaptoethanol. Bulk cultures consisted of 10^7 cells in 5 ml of media per well of a six-well cluster plate (Costar, Cambridge, Mass.). Microcultures consisted of 5 × 10^5 cells in 0.2 ml of media per well of a 96-well flat-bottomed microtiter plate (Costar). Virus-stimulated cultures were incubated with HSV at a multiplicity of infection of 1.0 PFU per cell calculated before inactivation. Influenza stimulation was achieved with 120 hemagglutination units of virus.

Interleukin 2 (IL-2) containing supernates from concanavalin A-stimulated rat splenocyte cultures (CAS) were prepared as previously described (14). IL-2 determinations were performed with the IL-2-dependent CTLL cell line (kindly provided by K. Smith, Dartford College, N.H.). Purified IL-2 was prepared according to previously published procedures (17).

Measurement of lymphocyte proliferation. The incorporation of tritiated thymidine ([^{3}H]TdR) into cellular DNA was used as a measure of lymphocyte proliferation. Typically, 0.5 μCi of [^{3}H]TdR (New England Nuclear Corp., Boston, Mass.) was added to the appropriate wells of the 96-well plate during the final 6 h of incubation. The cells were then harvested onto glass fiber filters (Skatron, Inc., Sterling, Va.) with a semiautomated cell harvester (Flow Laboratories, Inc., McLean, Va.). The filter papers were immersed in 0.5 ml of ScintiVerse E (Fisher Scientific Co., Fair Lawn, N.J.) and counted in an LS 7000 liquid scintillation spectropho-

---

* Corresponding author.

tometer (Beckman Instruments, Inc., Irvine, Calif.). Results are expressed as the mean value obtained from four replicate wells.

Negative depletion of the microculture wells was performed by transferring 100 µl of cells to V-bottomed Linbro trays (Flow). The cells were pelleted by centrifugation (200 × g, 5 min), and the supernatant fluid was discarded. The cells were suspended in 100 µl of the diluted antiserum and incubated for 45 min on ice. The cells were again pelleted, and the supernatant was discarded; the cells were then suspended in 100 µl of rabbit complement diluted 1/13 in cytotoxicity medium (Accurate Chemical and Scientific Corp., Westbury, N.Y.). The plates were incubated for 30 min at 37°C. Afterward the cells were washed with cytotoxicity medium and suspended in RPMI 1640 containing 0.5 µCi of [^{3}H]TdR. The cultures were incubated for 6 h and then harvested onto glass fiber filters.

Negative depletion of the bulk cultures was performed as previously described (10). Briefly, 10^7 splenocytes were suspended in specific antiserum and incubated for 45 min at 4°C. The cells were washed with cytotoxicity medium and suspended in complement. After being incubated at 37°C for 30 min, the cells were washed several times with cytotoxicity medium and then suspended in complete RPMI 1640. Viability of the treated cultures was determined by trypan blue exclusion. The number of cells per milliliter was adjusted after viability determinations were performed.

Coculture experiments. Suppressor-inducer cultures consisted of HSV-immune splenocytes or nylon wool-purified Thy 1^+ cells incubated in the presence of HSV antigens as described above. Nylon wool-purified cells were stimulated with HSV-infected resident peritoneal cells from syngeneic mice. In some experiments, the cells were treated with specific antiserum and complement before viral stimulation. Normal splenocytes were added as filler cells to some of these depleted cultures. Enough filler cells were added to the

TABLE 2. Lyt 1 depletion inhibited proliferation, whereas Lyt 2 depletion caused enhancement[a]

Treatment	[^{3}H]TdR incorporated (cpm)
None	18,125
Complement	18,650
Anti-Lyt 1.1	895[b]
Anti-Lyt 1.1 + IL-2	7,015[b]
Anti-Lyt 2	19,685[c]

[a] HSV-immune splenocytes were depleted of Lyt 1^+ or Lyt 2^+ cells before incubation with HSV. IL-2 (20 U) was added to some of the Lyt 1-depleted cultures at their onset. All cultures were incubated for 5 days at 37°C and then pulsed for 4 h with 0.5 µCi of [^{3}H]TdR. Each value represents the average for four replicate wells. Standard errors were 10% or less.
[b] Significantly different from the control wells at $P < 0.001$.
[c] Significantly different from the control wells at $P < 0.05$.

depleted cultures to replace the cells lysed by the antibody treatment. After 3 days, the suppressor-inducer cultures were irradiated (2,000 rads, X-irradiation), and then 10^5 cells were added to 4×10^5 HSV-stimulated, HSV-immune or 4×10^5 influenza-stimulated, influenza-immune splenocytes. These test cultures were incubated for 5 days, and the LPR was determined as described above.

Statistical analysis. The results presented in this paper are representative of experiments that were performed at least four times. In vitro assays were always performed in quadruplicate, and in vivo determination of delayed-type hypersensitivity responses involved at least five mice per treatment group. Data were analyzed with Student's t test and an analysis of variance.

RESULTS

Multiple cell types responded to HSV in the LPR. To assess the relative contribution of B cells, T cell subsets, and natural killer cells to stimulation by UV-inactivated HSV, nonimmune and HSV-immune splenocytes were incubated with antigen for 5 days and then depleted of various cell types by negative selection with antibody and complement. The remaining cells were pulsed with [^{3}H]TdR to assess their contribution to the LPR. Only antigen-stimulated immune lymphocytes responded significantly (Table 1), a response which peaked on day 5 (data not shown). It is apparent that the bulk of the proliferating immune cells were T cells, with around 10% expressing surface immunoglobulin and Ia. These were assumed to be B lymphocytes, but further characterization was not attempted. Of the T cell fraction, approximately 60% of the proliferating cells expressed the Lyt 1.1 marker, and approximately 40% of the cells were Lyt 2.1.

Regulation of the LPR to HSV. The part played by Lyt 1^+ and Lyt 2^+ cells in the LPR was further analyzed by depleting a given cell type from cultures before antigen stimulation and then by testing levels of [^{3}H]TdR incorporation after 5 days of culture. Treatment with anti-Lyt 1.1 and complement to remove Lyt 1^+ cells completely abrogated the LPR, but the response could be partially restored by the addition of exogenous lymphokines (Table 2). The partially restored LPR represented Lyt 2^+ proliferation (data not shown), and in fact, the level of [^{3}H]TdR incorporated by the Lyt 1^- cultures approximated that achieved by Lyt 2^+ cells in the undepleted cultures (compare Table 1 with Table 2).

When splenocytes were depleted of Lyt 2^+ cells, the level of [^{3}H]TdR incorporated by the remaining cells was not diminished but instead was significantly greater than that of

TABLE 1. Identification of the proliferating cells by using negative selection procedures[a]

Control	Immune cells + virus antiserum treatment	[^{3}H]TdR incorporated (cpm)	% Total
Normal + virus	None	1,748	
HSV immune	None	1,405	
	Complement	17,545	100.0
	Anti-Thy 1.2 + complement	1,713[b]	9.7
	Anti-Lyt 1.1 + complement	8,305[c]	42.1
	Anti-Lyt 2.1 + complement	10,942[c]	62.4
	Anti-immunoglobulin + complement	15,850	90.3
	Anti-IA^k + complement	15,650	89.2
	Anti-asialo GM$_1$ + complement	17,405	99.2

[a] HSV-immune splenocytes were incubated with UV-inactivated HSV for 5 days. The cells were then washed and incubated with specific antiserum and complement before pulsing with [^{3}H]TdR. Each value represents the average counts per minute for four replicate wells per treatment group. Standard errors were 10% or less. The percentage of the total was calculated as the level of [^{3}H]TdR incorporated by the treated culture divided by the [^{3}H]TdR incorporated by the complement control.
[b] Significantly different from the complement control at $P < 0.001$.
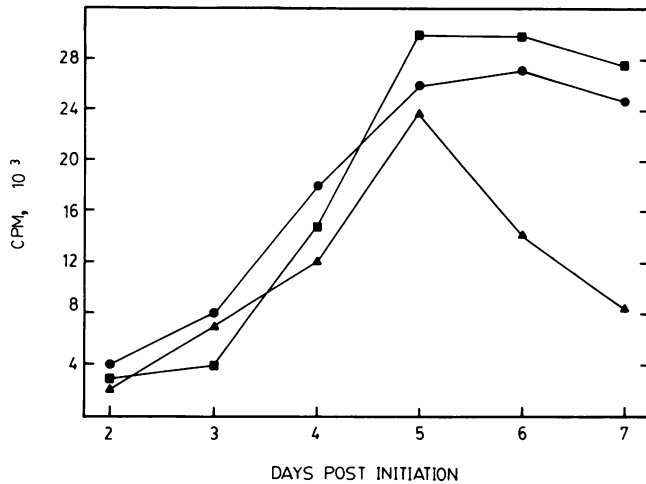[c] Significantly different from the complement control at $P < 0.01$.

FIG. 1. Time course of [^{3}H]TdR incorporation by depleted splenocyte cultures. Splenocytes were obtained from immune mice and were depleted of Lyt 2$^+$ (●) or IJ$^+$ (■) cells before culturing. Controls (▲) were treated with complement alone. [^{3}H]TdR incorporated by each culture was determined at daily intervals. Each point represents the mean for four replicates. Standard errors of the means were <10%.

the untreated controls (Fig. 1). Thus, in the absence of Lyt 2$^+$ cells, the remaining cells exhibited enhanced proliferation by day 5 of culture. One interpretation of these results is that an Lyt 2$^+$ suppressor cell regulated the LPR of other cells to the antigen. Further support for this hypothesis was obtained by removal of IJ$^+$ cells before culture. The IJ antigen is a well-accepted marker of certain subsets of suppressor T cells (2, 4). Removal of either Lyt 2$^+$ or IJ$^+$ cells provided a significant enhancement of [^{3}H]TdR incorporation (Fig. 1). This was particularly marked on days 6 and 7 of culture. The results support the role of Lyt 2$^+$ and IJ$^+$ suppressor cells regulating the LPR.

To further reveal the mechanism of suppression and to better identify the suppressor cell regulating the LPR to HSV, the following coculture experiments were performed. HSV-immune splenocytes were incubated with heat-inactivated HSV for 3 days and subsequently separated into various subpopulations based on adherence and antigen expression. The selected cells were then irradiated (2,000 rads, X-irradiation) and added to test cultures at their initiation. On day 5 of culture, the test cultures were pulsed

TABLE 3. Identification of a T suppressor cell in HSV-stimulated, HSV-immune splenocyte cultures^a

Cells added	[^{3}H]TdR uptake (cpm)
None	22,150
Plastic nonadherent	2,225^b
Nylon wool nonadherent	1,350^b
Anti-IJ^k	23,450
Anti-Thy 1.2	24,785
Anti-Lyt 1.1	4,505^b
Anti-Lyt 2.1	26,550

^{a} HSV-stimulated, HSV-immune splenocytes were incubated for 3 days and then depleted of various cell populations by using either adherence or antisera and complement. The remaining cells were then irradiated (2,000 rads), and 10^5 cells were added to 4 × 10^5 cells of the test cultures. The test cultures were incubated for 5 days, and [^{3}H]TdR uptake was assessed. Standard errors were <10%.
^{b} Significantly less than control at $P < 0.001$.

with [^{3}H]TdR and harvested as described above. As shown in Table 3, 3-day-old X-irradiated cells from HSV-stimulated, HSV-immune splenocyte markedly (up to 90%) suppressed the incorporation of [^{3}H]TdR by the test cultures. Thus, suppressor cells were neither plastic nor nylon wool adherent and expressed the Thy 1.2, Lyt 2.1, and the IJ antigens.

Although suppressor cells from antigen-stimulated immune cultures markedly suppressed the LPR, the same cells unstimulated or stimulated with influenza virus or antigen-stimulated normal splenocytes failed to inhibit the LPR in the test cultures (data not shown). This requirement for both in vivo activation and in vitro HSV-stimulation for suppressor cell induction resembled the requirements for HSV-specific cytotoxic T lymphocyte (CTL) induction (11).

Cellular requirements for suppressor cell induction. The next series of experiments was designed to identify the cellular requirements for suppressor cell induction. Before incubation with HSV, HSV-immune splenocytes were treated with specific antisera and complement. After 3 days of incubation with the virus, the cells were irradiated and added to test cultures at their initiation. The test cultures, consisting of unseparated HSV-immune splenocytes and HSV, were incubated for 5 days and pulsed with [^{3}H]TdR 6 h before harvesting. Nondepleted suppressor cell induction cultures developed suppressor activity which inhibited proliferation in the test cultures by 75%. Pretreatment of the suppressor induction cultures with anti-Lyt 1.1, -Lyt 2.1, or -IJ^k antisera and complement abrogated suppressor cell activity (Table 4). It was possible to restore suppressor activity in the Lyt 1.1- and IJ-depleted induction cultures by replacing the lysed cells with splenocytes from normal mice. In contrast, normal splenocytes could not restore suppressor activity to the Lyt 2-depleted cultures. Thus, only Lyt 2$^+$ cells needed to be expanded by antigen exposure in vivo (and in vitro) to generate suppression.

To further analyze the role of various cell types in suppressor cell induction, nylon wool-nonadherent cells (consisting of >99% Thy 1$^+$ cells) were used for the suppressor

TABLE 4. Cellular requirement for the induction of T suppressor cells in HSV-stimulated, HSV-immune splenocyte cultures^a

Cells added	[^{3}H]TdR uptake^b (cpm)	% Suppression^c
None	17,128	
Immune	4,181^d	75.6
Lyt 1$^-$	15,892	7.2
Lyt 1$^-$ + normal	9,237^d	46.1
Lyt 2$^-$	18,522	−8.1
Lyt 2$^-$ + normal	18,340	−7.1
IJ$^-$	20,228	−18.1
IJ$^-$ + normal	10,075^d	41.2

^{a} Suppressor cell induction cultures were prepared as described in the text and in the footnote to Table 7. Some of the cultures were treated with specific antiserum and complement before incubation with the virus. Normal splenocytes (2 × 10^7) were added to some of the Lyt 1-depleted cultures, and 10^7 normal cells were added to the Lyt 2-depleted cultures to replace the cells which were lysed by antibody and complement treatment. As before, the splenocytes were irradiated on day 3 and then added to the test cultures. Then 10^5 suppressor cells were added to 4 × 10^5 cells of the test culture.
^{b} The test cultures were pulsed with 0.5 μCi of [^{3}H]TdR for 6 h before harvesting onto glass fiber filters. Each value represents the mean of four replicates from a representative experiment. Standard errors were <10% of the mean in all cases.
^{c} Calculated as 1 − (experimental counts per minute/control counts per minute) × 100.
^{d} Significantly less than the control at $P < 0.05$.

TABLE 5. T cell requirement for the induction of T suppressor cells in HSV-stimulated, HSV-immune T cell cultures[a]

Cells added	[³H]TdR uptake[b] (cpm)	% Suppression[c]
None	27,328	
Complement treated	11,172[d]	59.1
Lyt 1 depleted	22,218	18.7
Lyt 1 depleted + normal T	14,738[d]	46.1
Lyt 2 depleted	28,238	-3.3
Lyt 2 depleted + normal T	28,780	-5.3
IJ[k] depleted	13,520[d]	50.5

[a] Nylon wool-purified, HSV-immune T cells were treated with antiserum and complement and then added to Thy 1⁻, HSV-stimulated residential peritoneal cells. Then 3×10^7 or 2×10^7 nylon wool-purified, normal T cells were added to some of the Lyt 1- and Lyt 2-depleted cultures to replace those cells lysed by the respective antiserum treatment. The cells were incubated for 3 days, irradiated, and added (20%) to HSV-immune, HSV-stimulated splenocyte cultures. These cultures were incubated for 5 days, and then 0.5 µCi of [³H]TdR was added in each well. Standard errors were <10%.
[b] Average counts per minute for four replicate wells.
[c] See Table 4, footnote c.
[d] Significantly less than control at $P < 0.01$.

cell induction cultures. Such cells generated suppressor activity when stimulated with HSV-treated peritoneal cells to act as antigen-presenting cells (APC) (Table 5). This cell population was depleted of T cells by anti-Thy 1 plus complement treatment. In the absence of added APC, antigen stimulation failed to generate suppression. Upon depletion of either Lyt 1⁺ or Lyt 2⁺ cells from these cultures before antigen stimulation, suppressor cell activity was not generated. However, unlike the situation with intact splenocyte responders, IJ depletion had no effect. This indicated that IJ⁺ cells were provided by the APC and were presumably required for suppressor cell induction. Support for this idea came from experiments in which the APC population was treated with anti-IJ plus complement before HSV exposure and use for induction (Table 6). After such treatment, suppression was not generated in the nylon wool-nonadherent responder population. Conversely, IA⁺ depletion of the peritoneal cell population actually enhanced the suppressor cell activity.

The question of the antigen specificity of the expression of suppressor cells was also addressed. For this purpose, antigen-induced suppressor populations were irradiated and added to two types of test cultures. The first were HSV-

TABLE 6. APC requirement for the induction of T suppressor cells in HSV-stimulated, HSV-immune T cell cultures[a]

Peritoneal cells added	[³H]TdR uptake (cpm)	% Suppression[b]
Complement	10,164[c]	40.7
Anti-IA	8,262[c]	51.8
Anti-IJ	19,518	-14.0
None	19,766	-15.4

[a] Nylon wool-purified, HSV-immune splenocytes were incubated with variously treated resident, Thy 1⁻ peritoneal cells from normal syngeneic mice. After 3 days of incubation with HSV, the cells were irradiated (2,000 rads), and 10^5 cells were added to 3×10^5 HSV-stimulated, HSV-immune splenocytes. On day 5 of culture, 0.5 µCi of [³H]TdR was added to each well for a 6-h pulse before harvesting onto glass fiber filters. Standard errors were <10%.
[b] Calculated as 1 − (experimental [³H]TdR uptake/control [³H]TdR uptake) × 100.
[c] Significantly less than the control at $P < 0.01$.

TABLE 7. T suppressor cells in HSV-stimulated, HSV-immune splenocyte cultures mediate nonspecific suppression[a]

Population added to virus-stimulated cultures	[³H]TdR uptake (cpm) from cultures stimulated with:	
	HSV	Influenza virus
None	23,501	15,380
Plastic nonadherent	9,660	6,379
Nylon wool nonadherent	4,169	2,050
Anti-immunoglobulin nonadherent	8,244	3,531
Anti-Thy 1,2 nonadherent	23,026	13,330

[a] HSV-stimulated, HSV-immune splenocytes were incubated for 3 days to induce suppressor cell activity. The cultures were then depleted of various subpopulations based on adherence or antigen expression. The remaining cells were then irradiated (2,000 rads) and subsequently added to HSV-stimulated or influenza-stimulated immune splenocyte cultures. The test cultures consisted of 4×10^5 immune splenocytes incubated with 10^5 cells from the suppressor induction cultures. The test cultures were incubated for 5 days, and [³H]TdR incorporation was determined. Each value represents the mean of four replicate wells. Standard errors were <10%.

immune splenocytes stimulated with HSV, and the second were influenza-immune splenocytes stimulated with influenza virus. It is readily apparent (Table 7) that the expression of suppression is nonspecific.

Finally, the possible mechanism of suppression was investigated. IL-2-containing supernatant fluids from CAS was added to the test cultures at the time of suppressor cell addition (Table 8). Though an optimal dosage of CAS stimulated [³H]TdR incorporation above the response of the control cultures, the addition of CAS failed to overcome the suppression mediated by the added suppressor cells. Increasing the concentration of CAS (10%, vol/vol) failed to overcome suppression (data not shown).

DISCUSSION

We have demonstrated that HSV-specific LPRs are regulated by suppressor cells, and we have, in addition, delineated some of the cell types involved in suppressor cell induction. Our results showed that HSV-specific LPR measures mainly the responses of two T cell subsets, Lyt 1⁺ and Lyt 2⁺. As in other systems (17), the actual LPR is driven by IL-2 produced by Lyt 1⁺ cells. Thus, in the absence of Lyt 1⁺ cells, LPR was almost eliminated, but the addition of extraneous IL-2 restored the LPR of Lyt 2⁺ cells. In the HSV system, unlike some others (5, 12), Lyt 2⁺ cells failed to produce their own IL-2 at least in sufficient quantity to drive proliferation. Upon removal of the Lyt 2⁺ cells before antigen stimulation, LPRs of the remaining cells were clearly elevated. This was taken as evidence for the presence of regulatory suppressor cells in the Lyt 2⁺ population. We

TABLE 8. IL-2 did not overcome suppressor cell activity[a]

Presence or absence of:		[³H]TdR uptake (cpm)
Suppressor cells	CAS	
−	−	17,888
−	+	20,283
+	−	3,188
+	+	2,569

[a] The 3-day stimulated, irradiated suppressor cells were added to HSV-stimulated, HSV-immune splenocyte test cultures with or without the addition of IL-2 containing 2.5% (vol/vol) CAS. Each value represents the mean of four replicate wells. The standard error of the mean was always <10%.

know from previous studies that CTLs are also Lyt 2^+ cells (11), so it was conceivable that suppression resulted in part from killing of proliferating antigen-expressing cells or APC (3). However, this idea was made unlikely by the observation that heat-inactivated viral antigen preparations elicited both the LPR and suppression. In contrast, heat-inactivated virus does not elicit HSV-specific CTL responses (14). Furthermore, IJ depletion of the cultures enhanced both the LPR and CTL activity.

In an attempt to further identify the cell types involved in suppression, experiments were done in which suppression was generated in one culture and those cells, after irradiation, were subsequently used to modulate the LPR of another antigen-stimulated test culture. After 3 days of antigen stimulation, potent suppressor cell activity was generated which could inhibit the induction of the LPR in test cultures by up to 90%. The cell type responsible for the suppression was an Lyt 2^+ IJ$^+$ cell, but several cell types appeared necessary for the generation of the suppressor cell. These included cells expressing the Lyt 2^+, the Lyt 1^+, and the IJ antigens. That all markers were expressed on a single suppressor cell precursor seemed unlikely. Rather, it appeared on the basis of indirect evidence that suppressor cell induction required the interaction of at least three cell types. Of the three putative cell types required for suppressor cell generation, only one, the Lyt 2^+, needed to be from HSV-immune animals. Thus, normal splenocytes could provide both the Lyt 1^+ and IJ$^+$ cells. The IJ-expressing cell required for suppressor cell induction was most likely an IJ$^+$ APC, because this cell was adherent, Thy 1^-, and present in peritoneal washes from nonimmune mice.

Cooperation between different cell subpopulations occurs in the induction of suppressor cells which regulate a variety of immune responses to noninfectious antigens (1, 2, 4). These interactions involve distinct T cell subpopulations and macrophages. Like the current study, the different T cell populations involved could be identified on the basis of Lyt antigen expression. Thus, suppressor inducer cells are Lyt 1^+, whereas suppressor effector and acceptor cells are Lyt 2^+ (1), and the APC are IJ$^+$ (2). The interactions of these cells also involve the production and presentation of soluble suppressor factors. Although some of these factors are antigen specific and genetically restricted in their action, the ultimate suppressor effect may be nonspecific.

It remains to be established how the various cell types interact in the HSV model to generate suppression and whether soluble factors are involved. Elsewhere we have shown that supernatant fluids from HSV-stimulated, HSV-immune splenocyte cultures suppress the LPR to HSV (D. W. Horohov, R. N. Moore, and B. T. Rouse, Fed. Proc. 43:1608, 1985). The suppressive supernatant fluid contains multiple suppressor activities, one of which is antigen specific. However, as in the current study, the ultimate suppressor effect appears to be mediated nonspecifically. Thus, both the suppressor cell and the soluble factors generated in the HSV-stimulated cultures suppress the LPRs to influenza. It is not known how either suppressor mechanism acts. Possible mechanisms of suppression include (i) interference with APC (3), (ii) the neutralization of factors, such as IL-2 produced by helper cells (6), and (iii) the release of mediators that inhibit T cell activation or division (9). Suppression of HSV-specific lymphoproliferation does not require CTL activity in the cultures and thus does not appear to involve lysis of the stimulatory cells. In conflict with the second mechanism and in support of the third alternative is our observation regarding the inability of exogenous IL-2 to

abrogate suppression by the Lyt 2^+ IJ$^+$ cells. Thus it appeared unlikely that suppression was the result of limiting amounts of growth factor. It appeared instead that suppression, probably mediated by one of the nonspecific soluble factors, was due to direct interference with the proliferating population. Similar mechanisms have been described in other systems (18).

It is important to understand how the suppressor cell system is activated and expresses its activity in vitro because clues may emerge as to manipulation of the system that will prove of value in vivo. For example, suppressor cells could serve to inhibit protective aspects of immunity before the development of recrudescent disease. There is some evidence that helper-to-suppressor T lymphocyte ratios do change around the time of recrudescence in humans (15), and recently in the guinea pig model of HSV-2, suppressor cells were demonstrated in the spleens of animals undergoing recrudescence (7). Indeed, if suppressor cells and their products are involved in modulated immunity to HSV in humans, this could provide a useful target for treatment aimed at breaking the cycle.

LITERATURE CITED

1. Asherson, G. L., V. Colizzi, and M. A. Zembala. 1984. The structure and biological action of antigen-specific T suppressor factor, p. 79–95. In M. J. Taussig (ed.), T cell hybridomas. CRC Press, Boca Raton, Fl.
2. Dorf, M. E., and B. Benacerraf. 1984. Suppressor cells in immunoregulation. Annu. Rev. Immunol. 2:127–158.
3. Fitch, F. W., H. D. Engers, J. C. Cerottini, and K. T. Brunner. 1976. Generation of cytotoxic T lymphocytes in vitro. VII. Suppressive effect of irradiated MLC cells on CTL response. J. Immunol. 116:716–723.
4. Germain, R. N., and B. Benacerraf. 1981. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. Scand. J. Immunol. 13:1–10.
5. Guerne, P.-A., P.-F. Piguet, and P. Vassalli. 1984. Production of interleukin 2, interleukin 3, and interferon by mouse T lymphocyte clones of Lyt 2^+ and 2^- phenotype. J. Immunol. 132:1869–1871.
6. Gullberg, M., and E.-L. Larsson. 1982. Studies on induction and effector functions of concanavalin A-induced suppressor cells that limit TCGF production. J. Immunol. 128:746–750.
7. Iwasaka, T., J. F. Sheridan, and L. Aurelian. 1983. Immunity to herpes simplex virus type 2: recurrent lesions are associated with the induction of suppressor cells and soluble suppressor factors. Infect. Immun. 42:955–964.
8. Kirchner, H. 1982. Immunobiology of infection with herpes simplex virus. In J. L. Melnick (ed.), Progress in Medical Virology, vol. 13. S. Karger, New York.
9. Kramer, M., and U. Koszinowski. 1982. T cell-specific suppressor factor(s) with regulatory influence on interleukin 2 production. J. Immunol. 128:784–790.
10. Larsen, H. S., M.-F. Feng, D. W. Horohov, R. N. Moore, and B. T. Rouse. 1984. Role of T-lymphocyte subsets in recovery from herpes simplex virus infection. J. Virol. 50:56–59.
11. Lawman, M. J. P., B. T. Rouse, R. J. Courtney, and R. D. Walker. 1980. Cell-mediated immunity against herpes simplex induction of cytotoxic T lymphocytes. Infect. Immun. 27:133–139.
12. Miller, R. A., and O. Stutman. 1982. Use of positively selected Lyt 2^+ mouse splenocytes to examine interleukin 2 secretion in responses to alloantigens and to TNP-modified syngeneic cells. Cell. Immunol. 68:114–127.
13. Rouse, B. T. 1984. Role of adaptive immune defense mecha-

nisms in herpes simplex resistance, p. 131–144. *In* B. T. Rouse and C. Lopez (ed.), Immunobiology of herpes simplex virus infection. CRC Press, Boca Raton, Fla.

14. **Schmid, D. S., and B. T. Rouse.** 1983. Cellular interactions in the cytotoxic T lymphocyte response to herpes simplex virus antigens. Differential antigen activation requirements for the helper T lymphocytes and cytotoxic T lymphocyte precursors. J. Immunol. **131:**479–484.

15. **Sheridan, J. F., A. D. Donnenberg, L. Aurelian, and D. J. Elpern.** 1982. Immunity to herpes simplex virus type 2. IV. Impaired lymphokine production during recrudescence correlates with an imbalance in T lymphocyte subsets. J. Immunol.

129:326–331.

16. **Shillitoe, E. J., J. M. A. Wilton, and T. Lehner.** 1977. Sequential changes in cell-mediated immune responses to herpes simplex virus after recurrent herpetic infections in humans. Infect. Immun. **18:**130–137.

17. **Smith, K. A.** 1980. T cell growth factor. Immunol. Rev. **51:**337–352.

18. **Zembala, M. A., G. L. Asherson, B. M. B. James, V. E. Stein, and M. C. Watkins.** 1982. Anti-hapten T suppressor factor acts through an I-J,[+] Ly 1-2[+] T acceptor cell that releases a non-specific inhibitor of the transfer of contact sensitivity when exposed to antigen. J. Immunol. **129:**1823–1829.