Regulation of Herpes Simplex Virus-Specific Cell-Mediated Immunity by a Specific Suppressor Factor

DAVID W. HOROHOF,[†] JOHN H. WYCKOFF III, ROBERT N. MOORE, AND BARRY T. ROUSE*

Department of Microbiology, Colleges of Veterinary Medicine and Liberal Arts, University of Tennessee, Knoxville, Tennessee 37996-0845

Received 1 November 1985/Accepted 27 January 1986

Our study was designed to investigate the nature of an antigen-specific suppressor factor generated by antigen-stimulated herpes simplex virus (HSV)-immune splenocytes. Factor SF-200, a 90,000- to 100,000-dalton fraction obtained after Sephacryl gel filtration, suppressed the generation of HSV-specific cytotoxic T-lymphocyte and lymphoproliferative responses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of SF-200 indicated that it contained an I-J⁺, anti-idiotypic protein. It was possible to adsorb the suppressor activity of SF-200 to an anti-I-J immunoaffinity column. The suppressor activity could be eluted from the immunoaffinity column with a low-pH buffer. The acid-eluted material was determined to be both I-J⁺ and reactive with anti-HSV antiserum by Western blot analysis. Both SF-200 and the I-J⁺ suppressor activity suppressed only HSV-specific cell-mediated immunity responses. However, it was possible to generate nonspecific suppressor activity by incubating the I-J⁺ suppressor factor with Lyt 1⁺ splenocytes from HSV-immune mice. The implication of these results with respect to the model for a suppressor cell circuit regulating HSV-specific cell-mediated immunity responses is discussed.

Immune responsiveness is regulated by suppressor cell circuits composed of various populations of macrophages and T lymphocytes (2, 12, 17, 18). Soluble factors transmit the suppressogenic signals between the T-cell subsets (1, 41) and mediate the ultimate suppressor effect (34, 42, 55). These factors may be either antigen specific or nonspecific in their action. Whereas the nonspecific factors contain neither genetic restriction elements nor receptor structures (4, 28), the antigen-specific factors possess either idiotypic antigen-binding sites or anti-idiotypic sites as well as express various genetic restriction elements (3, 16, 51, 52). The antigen-specific suppressor factors appear to be composed of separate subunits joined by disulfide bonds. In such cases, the idiotypic or anti-idiotypic site is present on one subunit, whereas the genetically restricted element (such as I-J) is found on another subunit (3, 15, 16, 31, 52). However, some suppressor factors consist of a single chain that expresses both I-J determinants and antigen-binding activity (24, 26).

The above information regarding the characterization and biological activity of suppressor cascades and suppressor factors has resulted from studies of immune responses to haptens, alloantigens, and other noninfectious antigens. Nevertheless, suppressor mechanisms apparently regulate the immune responses to a variety of infectious agents, including bacteria (10, 36, 40), fungi (38), protozoans (8, 37, 46, 47), helminths (14, 19, 44), and viruses (7, 9, 20, 21, 23, 32, 33, 35, 39, 43, 45). The role that these suppressor cells have in the disease process remains unclear. For example, both suppressor cells and factors were shown to influence various aspects of immune responsiveness to herpes simplex virus (HSV) (21, 23, 39, 45), but the implication of such suppression in the pathogenesis of herpesvirus infections remains to be established. One attractive proposal was that suppressor cell activity played an important role in recrudescence of herpetic disease (23, 48, 49). Further investi-

gation of this concept will require better-defined suppressor cell populations and their soluble factors. In this report, we further investigate the nature of the suppressor factors which regulate HSV-specific responses and describe an antigen-specific suppressor factor which binds both I-J- and HSV-specific antibodies. Incubation of this specific factor with HSV-stimulated, HSV-immune Lyt 1⁺ splenocytes resulted in the production of nonspecific suppressor activity.

MATERIALS AND METHODS

Virus preparations. HSV type 1 strain KOS was propagated in HEP-2 cells as described previously (30). The viral stock had an infectivity titer of 4×10^8 PFU/ml. UV-inactivated virus 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 titer to fewer than 10^2 PFU/ml. Influenza virus strain A/PR8/34, provided by J. Bennick (Wistar Institute of Anatomy and Biology, Philadelphia, Pa.), was propagated in embryonated chicken eggs.

Cells. Strain L929 cells (*H-2^k*) were obtained from the American Type Culture Collection (Rockville, Md.), 3T3 strain A31 cells (*H-2^d*) were obtained from Ray Tennant (Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.), and EL4 thymoma cells (*H-2^b*) were obtained from M. Hilfiker (Cleveland Clinic Foundation, Cleveland, Ohio). All cells were cultured in McCoy 5A medium supplemented with 5% donor calf serum (GIBCO Laboratories, Grand Island, N.Y.). The cells and viral stocks were routinely tested for mycoplasma contamination by the method of Kaplan et al. (25).

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. C57BL/6 (*H-2^b*) mice were purchased from Cumberland View Farms, Clinton, Tenn. The mice received a single 0.1-ml intraperitoneal injection of 10^6 PFU of HSV or 160 hemagglutinating units of influenza virus 4 weeks prior to use. In some experiments, the mice

* Corresponding author.

[†] Present address: Division of Virology, Federal Drug Administration, Bethesda, MD 20892.

received an intravenous injection of 0.1 ml of a 1/5 dilution of anti-asialo GM₁ antiserum (Wako Chemicals, Dallas, Tex.) 18 h prior to sacrifice. This treatment effectively eliminated natural killer cell activity in the splenocyte cultures (6).

The preparation of single-cell suspensions of splenocytes has been described elsewhere (30). The splenocytes were cultured in RPMI-1640 (GIBCO) 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} M 2-mercaptoethanol (complete medium). Bulk cultures consisted of 10^7 cells in 5 ml of medium per well of a six-well cluster plate (Costar, Cambridge, Mass.). Microculture consisted of 5×10^5 cells in 0.2 ml of medium per well of a 96-well flat-bottomed microtiter plate (Costar). HSV-stimulated cultures were incubated with HSV at a multiplicity of infection of 1.0 PFU per cell calculated before inactivation. Influenza virus-stimulated cultures were incubated with 40 hemagglutinating units in serum-free medium for 15 min and were then added to medium containing fetal calf serum.

Measurement of lymphocyte proliferation. The incorporation of tritiated thymidine into cellular DNA was used as a measure of lymphocyte proliferation. Typically, 0.5 µCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.) was added to the wells of the 96-well plate during the final 6 h of incubation. The cells were then harvested onto glass fiber filters (Skatron Inc., Steding, 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 a Beckman LS 7000 liquid scintillation spectrophotometer. Results are expressed as the mean value obtained from four replicate wells.

CTL activity. The cytotoxic T-lymphocyte (CTL) activity of bulk cultures and microcultures was assessed as described previously (21, 30). A total of 10^4 ⁵¹Cr-labeled, virus-infected, syngeneic or allogeneic cells were added to each of the four replicate wells. The targets were prepared as described by Lawman et al. (30). The plates were centrifuged ($200 \times g$; 2 min) and incubated for 3 h at 37°C. The ⁵¹Cr released from the lysed cells into the supernatant fluid was then measured. The percent specific cytotoxic activity was determined as [(experimental release - spontaneous release)/(total release - spontaneous release)] $\times$ 100. Each determination was performed in quadruplicate. The spontaneous release did not exceed 15% of the total release. The effector cell/target cell ratio in the microculture wells approximated 12:1 (data not shown).

Cell separation. Murine-derived anti-Thy 1.2, anti-Lyt 1.1, anti-Lyt 2.1, anti-I-J^k, anti-I-A^k, and anti-I-E^k antisera and complement were purchased from Cedarlane Laboratories (Accurate Chemical and Scientific Corp., Westbury, N.Y.). Cell separation was achieved by negative selection of splenocytes as described elsewhere (29). 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 medium. The viability of the treated cultures was determined by trypan blue exclusion. The number of cells per milliliter was adjusted after viability determinations were performed.

Preparation of suppressor factor. Supernatant fluids from HSV-immune cultures were prepared as described previously (21). Briefly, supernatant fluids from HSV-stimulated, HSV-immune splenocyte cultures were harvested on day 5,

dialyzed overnight against RPMI-1640, filter sterilized (0.2-µm-pore Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.), and stored frozen at -70°C. Serum-free supernatants were prepared for biochemical fractionation by incubating the splenocytes for 3 days in complete medium, pelleting the cells, and then suspending them in serum-free RPMI-1640. Following an additional incubation for 48 h, the serum-free conditioned medium was concentrated by ultrafiltration on an Amicon PM-10 membrane and dialyzed overnight against phosphate-buffered saline (PBS) containing 0.05% polyethylene glycol. Gel filtration chromatography was performed with Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, N.J.). The column dimensions were 100 by 1.5 cm, and the column was equilibrated with PBS containing 0.05% polyethylene glycol at a flow rate of 8 ml/h at 4°C. Molecular weight standards of 150,000, 65,000, and 120,000 were used to calibrate the column. A 1-ml portion of the concentrated serum-free suppressive supernatant fluid was applied to the column and eluted with PBS containing 0.05% polyethylene glycol. Fractions (2 ml) were collected, dialyzed overnight against RPMI-1640, filter sterilized, and assayed at final concentrations of 2.5 to 25% by the microculture method.

Immunoaffinity columns were prepared by published procedures (5). Immunoglobulins were purified from antiserum samples by affinity chromatography with protein A conjugated to Sepharose 4B (Pharmacia). Antibodies were then attached to Sepharose support beads with cyanogen bromide-activated Sepharose 4B (Pharmacia). Samples to be applied to the immunoaffinity columns were first dialyzed against PBS containing 0.5 M NaCl. The samples were mixed with the column gel overnight on a rocking platform at 4°C. The mixture was then poured into a 10-ml disposable column (Bio-Rad Laboratories, Rockville Centre, N.Y.) and washed with PBS containing 0.5 M NaCl until no detectable protein was eluted from the column. The bound material was removed from the immunoaffinity column by the application of a low-pH glycine buffer. The eluted fractions were collected directly into 0.2 M Tris base (pH 8.2). The collected fractions were dialyzed against PBS and then RPMI-1640 prior to the assay.

Dialyzed suppressor factor from the anti-I-J immunoaffinity column (HSV-SF) was added to HSV-stimulated, HSV-immune, and normal splenocyte cultures. In some experiments, the cultures were depleted of various subpopulations of splenocytes before being incubated with virus and suppressor factor. To test for the presence of suppressor-acceptor cells, we pretreated some mice with 20 mg of cyclophosphamide per kg 48 h prior to incubating their splenocytes with virus and suppressor factor. Supernatant fluids from these various splenocyte cultures were collected on day 3 and later tested for nonspecific suppressor activity by assaying against both HSV-specific and influenza virus-specific CTL induction.

SDS-PAGE and Western blotting. The methods used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described by Compton and Courtney (11). All running gels were 7.5% acrylamide with 4% stacking gels. Approximately equal amounts of protein (40 µg) in a sample volume of 50 µl were added to each well. Proteins were resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.), and immunoblotted by published procedures (54). As the primary antiserum, we used anti-I-J^k, anti-I-A^k, or anti-I-E^k antisera (Cedarlane Laboratories), rabbit anti-HSV antiserum, or normal rabbit serum (kindly

provided by Janet Lathey, Department of Microbiology, University of Tennessee, Knoxville). Specific antibody binding after subsequent washing was detected with ^{125}I -labeled protein A. Images of the resultant immunoblots were then developed through autoradiography. For molecular weight determinations, HSV-SF and molecular weight standards were submitted to SDS-PAGE, and the gel was stained directly with an ultrasensitive silver stain (57).

Statistical analysis. The results presented are representative of experiments that were performed at least four times. In vitro assays were always performed in quadruplicate. Data were analyzed by Student's *t* test and by an analysis of variance.

RESULTS

Generation and demonstration of the antigen-specific suppressor factor. Supernatant fluids from HSV-stimulated, HSV-immune T-lymphocyte cultures significantly inhibited HSV-specific CTL induction and lymphoproliferation when added to test cultures. Whereas the unfractionated suppressive supernatant also inhibited responses to influenza virus (Table 1), gel filtration on Sephacryl S-200 revealed a peak with an apparent molecular weight of 90,000 to 100,000 (SF-200) which inhibited both CTL induction and lymphoproliferation in an antigen-specific and genetically restricted fashion. Thus, the cell-mediated immunity responses to HSV were suppressed, but the same responses to influenza virus were barely affected. Likewise, the HSV-

TABLE 1. Suppression of HSV-specific cell-mediated immunity by a suppressor factor produced by HSV-stimulated, HSV-immune T lymphocytes^a

Sample tested	Cells tested ^b	% Specific CTL activity	Tritiated thymidine uptake (cpm)
None	CHSV	37.5 ± 4.4	24,235 ± 2,115
	CFLU	18.9 ± 2.8	18,245 ± 1,645
	BHSV	24.8 ± 3.2	31,245 ± 2,077
IVS	CHSV	6.5 ± 4.1	4,350 ± 633
	CFLU	3.1 ± 6.0	2,225 ± 189
	BHSV	11.3 ± 4.2	9,750 ± 115
IVSΔ	CHSV	35.0 ± 4.7	24,580 ± 1,866
	CFLU	19.9 ± 3.2	18,090 ± 1,774
	BHSV	23.5 ± 4.0	27,655 ± 2,225
SF-200	CHSV	11.2 ± 2.6	3,575 ± 490
	CFLU	19.3 ± 3.5	17,250 ± 220
	BHSV	24.0 ± 2.1	29,775 ± 307

^a Splenocytes from HSV-immune mice were incubated with HSV antigens for 3 days and then depleted of macrophages and B cells by passage over nylon wool columns. The nonadherent cells were determined to be greater than 95% Thy 1.2⁺ by antibody and complement-mediated cytotoxicity. Thy 1⁺ cells were then cultured an additional 3 days in serum-free medium. Afterwards, the cells were pelleted by centrifugation, and the supernatant (IVS) was assayed for suppressor activity. In some experiments, the supernatant was heated at 60°C for 15 min IVSΔ. Serum-free supernatants were concentrated 1,000-fold and applied to a Sephacryl S-200 gel filtration column. Those fractions which suppressed HSV-specific but not influenza virus-specific CTL induction were pooled (SF-200). All supernatant fluids and column fractions were assayed at a final concentration of 20% (vol/vol) after dialysis overnight against serum-free RPMI-1640. Data are expressed as the mean ± standard deviation.

^b CHSV, HSV-stimulated, HSV-immune splenocytes from C3H/HeJ mice; CFLU, influenza virus-stimulated, influenza virus-immune splenocytes from C3H/HeJ mice; BHSV, HSV-stimulated, HSV-immune splenocytes from C57BL/6 mice.

TABLE 2. Antigen-specific suppression of HSV-specific cell-mediated immunity by the I-J⁺ suppressor activity of SF-200^a

Sample tested	Cells tested ^b	% Specific CTL activity	Tritiated thymidine uptake (cpm)
None	HSV	37.5 ± 5.2	24,235 ± 2,135
	FLU	18.9 ± 2.2	18,245 ± 1,260
I-J ⁻	HSV	43.3 ± 6.5	22,755 ± 2,305
	FLU	18.5 ± 2.2	18,880 ± 1,655
I-J ⁺	HSV	3.2 ± 2.2	6,445 ± 702
	FLU	19.0 ± 2.4	18,555 ± 225
Immunoglobulin ⁻	HSV	2.4 ± 2.4	7,257 ± 577
	FLU	19.7 ± 2.1	17,987 ± 1,855
Immunoglobulin ⁺	HSV	42.1 ± 6.0	24,895 ± 2,366
	FLU	21.5 ± 3.2	18,535 ± 966

^a SF-200 fractions were concentrated and applied to anti-I-J^k and anti-immunoglobulin immunoaffinity columns. The unadsorbed fractions (I-J⁻, immunoglobulin⁻) and those fractions collected after elution with a pH buffer (I-J⁺, immunoglobulin⁺) were concentrated 15-fold and dialyzed against PBS and then RPMI-1640 before the assay. All samples were tested at a final concentration of 10% (vol/vol). Each determination was performed at least four times. Data are expressed as the mean ± standard deviation.

^b HSV-immune, HSV-stimulated (HSV) or influenza virus-immune, influenza virus-stimulated (FLU) C3H/HeJ splenocytes were incubated in vitro with the column eluates for 5 days. Both the CTL and lymphoproliferative responses of the cultures were assessed as described in Materials and Methods. The results reported are from a representative experiment.

specific responses of C57BL/6 (*H-2^b*) mice were unaffected by C3H/HeJ (*H-2^k*)-generated SF-200. Furthermore, this suppressor activity was heat labile, suggesting a protein composition. The suppressor activity was not generated by antigen-stimulated normal splenocytes or by unstimulated immune cells (21).

Characterization of the antigen-specific suppressor factor. Antigen-specific suppressor factors frequently possess determinants which react with anti-I-J antibodies (56). The exposure of SF-200 to an anti-I-J^k immunoaffinity column resulted in the removal of the suppressor activity (Table 2). In contrast, exposure to an anti-immunoglobulin immunoaffinity column failed to reduce the suppressor activity in SF-200. The material which bound to the anti-I-J column was eluted by application of a low-pH glycine buffer, dialyzed, and tested for suppressor activity against HSV- and influenza virus-specific CTL induction. The acid eluate from the anti-I-J column contained the HSV-specific suppressor activity (HSV-SF) (Table 2). Furthermore, SDS-PAGE and Western blotting demonstrated that this eluate also reacted with the anti-I-J antiserum but not with either the anti-I-A or the anti-I-E antiserum (Fig. 1A, B, and D). Silver staining of the gel used to determine molecular weight demonstrated four protein bands in the 55,000- to 70,000-molecular-weight region contained within HSV-SF (Fig. 2, lane 6). The highest-molecular-weight band corresponded to the single I-J⁺ band in the Western blots. The other bands in the silver-stained gel were presumed to represent degradation products as a result of the acid elution from the anti-I-J column prior to SDS-PAGE because they no longer bound anti-I-J antibodies in subsequent Western blotting. HSV-SF samples treated with 2-mercaptoethanol were incompletely reduced (Fig. 2, lane 7), precluding further interpretation of the polypeptide nature of this factor.

In addition to expressing I-J determinants, antigen-specific suppressor factors often bear anti-idiotypic determinants

FIG. 1. Western blot analysis of the HSV-specific suppressor factor. Anti-I-J immunoaffinity column fractions were submitted to SDS-PAGE. Following electrophoresis, proteins were electroeluted from the gels onto nitrocellulose and then incubated with anti-I-A antiserum (panel A), anti-I-E antiserum (panel B), normal rabbit serum (panel C), anti-I-J antiserum (panel D), or anti-HSV antiserum (panel E). Lane 1 is the anti-I-J⁺ immunoaffinity column-purified material, and lane 2 is the drop-through from the anti-I-J⁺ immunoaffinity column. KD, Kilodaltons.

which react with specific antibodies against the inducing antigen (3, 51). We next determined whether I-J⁺ HSV-SF also reacted with a specific rabbit anti-HSV antiserum which has been shown to share idiotypes with both human and murine anti-HSV antisera (J. R. Lathey, B. T. Rouse, D. E. Wiley, and R. J. Courtney, Immunology, in press). Through Western blotting, a single band in I-J⁺ HSV-SF was detected with the anti-HSV antiserum but not with normal rabbit serum (Fig. 1C and D). This band directly corresponded to both the I-J⁺ band in the anti-I-J Western blot (Fig. 1D) and the 68,500-molecular-weight band in the silver-stained gel (Fig. 2, lane 6). Together, these results indicate that the I-J⁺ suppressor activity reacted with the HSV-specific antiserum.

Induction of nonspecific suppressor activity in HSV-immune, HSV-stimulated splenocyte cultures by I-J⁺, HSV-specific suppressor activity. Anti-idiotypic suppressor factors in the hapten models are not themselves responsible for suppression but instead induce other cells to produce the eventual suppressor molecule (2, 34). We wished to establish if suppression induced by concentrated eluates from the anti-I-J immunoaffinity column (HSV-SF) was mediated directly or depended on the activity of some auxiliary mechanism. To test this, we treated virus-stimulated or unstimulated HSV- or influenza virus-immune splenocyte populations (acceptor population) with HSV-SF for 3 days, after which the culture supernatants were collected and assayed against both HSV- and influenza virus-specific CTL induction (Table 3). As reported elsewhere, low levels of suppressor activity were detectable after 3 days of HSV stimulation of the HSV-immune splenocytes (21). However, the addition of HSV-SF to the HSV-stimulated, HSV-immune acceptor population resulted in an increase in the nonspecific suppressor activity in the supernatant fluids. In the absence of HSV stimulation, HSV-SF failed to induce the production of the nonspecific suppressor activity by the acceptor population (Table 3, group F). Furthermore, such factors were not generated by antigen-stimulated, HSV-SF-

treated normal acceptor populations or by influenza virus-primed splenocytes.

The nature of the cell type responding to the HSV-SF stimulus was further investigated. Levels of suppression were markedly reduced if the acceptor population was taken from mice treated 2 days previously with cyclophosphamide (Table 4). Furthermore, if Lyt 1⁺ or I-J⁺ but not Lyt 2⁺ cells were removed from the acceptor population prior to the addition of HSV-SF and antigenic stimulation, the nonspe-

FIG. 2. SDS-PAGE of bulk suppressive supernatant and fractionated components. Protein samples (40 μ g) were submitted to SDS-PAGE, and the gel was silver stained. Lanes: 1, bulk supernatant; 2, S-200 fraction; 3 and 4, drop-through and eluate, respectively, from the anti-immunoglobulin immunoaffinity column; 5 and 6, drop-through and eluate (HSV-SF), respectively, from the anti-I-J immunoaffinity column; 7, HSV-SF after treatment with 2-mercaptoethanol. The migration of the molecular weight markers is indicated, and the values are expressed in kilodaltons.

TABLE 3. Production of nonspecific suppressor factors by HSV-SF-treated splenocytes^a

Acceptor population	Virus added	HSV-SF added	CTL induction culture tested	% Specific CTL activity	% Suppression
A. None	None	—	HSV specific	33.6 ± 6.6	— ^b
B. HSV immune	HSV	—	HSV specific	34.0 ± 6.0	0
C. HSV immune	HSV	+	HSV specific	21.0 ± 3.0	37.5 ^c
D. FLU immune	HSV	+	HSV specific	31.2 ± 5.0	7.1
E. None	None	—	FLU specific	25.8 ± 5.1	—
F. HSV immune	FLU	+	FLU specific	26.2 ± 4.2	0
G. HSV immune	HSV	+	FLU specific	15.8 ± 2.9	38.8 ^c
H. FLU immune	FLU	+	FLU specific	23.3 ± 4.7	9.7

^a HSV-SF was added to various splenocyte cultures. After incubation for 3 days, the supernatant fluids were collected and assayed for suppressor activity with HSV-specific C3H/HeJ and influenza virus (FLU)-specific C57BL/6 CTL induction cultures. The supernatant fluids were added to the cultures at a final concentration of 25% (vol/vol). The percent specific CTL activity of the test cultures was calculated as described in Materials and Methods. Percent suppression = [1 - (specific CTL activity of HSV-SF-treated induction cultures/specific CTL activity of untreated CTL induction cultures)] × 100. Data represent four experiments and are expressed as the mean ± standard deviation.

^b —, Untreated controls for each CTL induction culture tested.

^c Significantly different from the untreated controls at $P < 0.01$.

sific suppressor factor was not produced. These results indicate that the acceptor cell necessary for the production of the nonspecific suppressor activity following exposure to HSV and HSV-SF was I-J⁺, Lyt 1⁺2⁻, and cyclophosphamide sensitive.

DISCUSSION

The in vitro stimulation of HSV-immune splenocytes with HSV antigens results in the production of a suppressive supernatant (21). This suppressive supernatant contained both nonspecific and HSV-specific soluble suppressor fac-

tors which could be separated from each other by gel filtration. In this report, we further characterized this HSV-specific suppressor activity and determined its relationship to the nonspecific suppressor activity present in the suppressive supernatant.

Antigen-specific suppressor factors frequently bear I-J determinants (56) and thus can be bound by anti-I-J antibodies, allowing the selective adsorption of these molecules. Since it was possible to selectively adsorb out as well as recover the HSV-specific suppressor activity from an anti-I-J immunoaffinity column, it appears that the HSV-specific suppressor factor bears I-J determinants. Western blot analysis of this immunoaffinity-purified suppressor material (HSV-SF) demonstrated a component which reacts with both I-J- and HSV-specific antisera (Fig. 1D and E) and has an apparent molecular weight of 68,500. The other bands observed in Fig. 2, lane 6, are most likely HSV-specific suppressor factor(s), either of the same species as the 68,500-dalton, I-J⁺ HSV-SF or of other species which have acid-labile I-J determinants and presumably do not function after immunoaffinity purification.

It is noteworthy that although HSV-SF was murine derived and a T-cell factor, we were able to detect this molecule with a rabbit anti-HSV antiserum, therefore indicating a sharing of idiotype between mice and rabbits. Idiotypic cross-reactions between different animal species, including mice and rabbits, have been reported for a viral antigen (27). Furthermore, it has been demonstrated in our laboratory that a murine-derived monoclonal antibody against glycoprotein D of HSV bears an idiotype which is also found in rabbit, murine, and human antisera against HSV (Lathey et al., in press). Such is the case with the rabbit anti-HSV antiserum used in this communication.

The reactivity of the suppressor factor with the anti-HSV antiserum indicates several possibilities regarding the composition of HSV-SF and its mechanism of antigenic specificity. Two possibilities are that the suppressor activity represents either a viral protein or a host-derived protein which binds viral proteins. Alternatively, the reactivity of the factor with the anti-HSV antiserum could be a result of the presence of anti-idiotypic determinants on the suppressor factor. We favor the latter hypothesis for several reasons. It seems unlikely that the suppressor activity is mediated by a virus-encoded protein, since HSV does not usually infect and persist in lymphocytes and since none of its proteins have been shown to be immunosuppressive. Like-

TABLE 4. Production of nonspecific suppressor factors by Lyt 1⁺ splenocytes treated with the I-J⁺, HSV-specific suppressor factor^a

Supernatant from HSV-SF-treated acceptor population	CTL induction culture tested	% Specific CTL activity	% Suppression
None	HIV	33.6 ± 5.9	— ^b
HI	HIV	34.0 ± 5.0	0.0
HIV	HIV	21.0 ± 4.1	37.5 ^c
HIV Lyt 1 ⁻	HIV	30.0 ± 5.5	10.7
HIV Lyt 2 ⁻	HIV	22.2 ± 3.8	33.9 ^c
HIV I-J ⁻	HIV	32.4 ± 6.2	3.6
HIV CY	HIV	29.6 ± 4.5	11.9
None	FIV	25.8 ± 4.1	—
HI	FIV	24.0 ± 3.9	7.0
HIV	FIV	15.8 ± 4.8	38.8 ^c
HIV Lyt 1 ⁻	FIV	23.3 ± 3.5	9.7
HIV Lyt 2 ⁻	FIV	16.6 ± 4.1	35.7 ^c
HIV I-J ⁻	FIV	23.3 ± 3.1	9.7
HIV CY	FIV	21.2 ± 2.8	17.8

^a I-J⁺ effluents from immunoaffinity column-purified SF-200 (HSV-SF) were added to various splenocyte cultures. Some of the splenocyte populations were pretreated with anti-Lyt 1.1, anti-Lyt 2.1, or anti-I-J^k antiserum and complement prior to the addition of the HSV-specific suppressor factor. This suppressor factor was added to these cultures at a final concentration of 2.5% (vol/vol). On day 3 of culturing, the supernatant fluids were harvested and tested for suppressor activity against CTL induction as described in Table 3, footnote a. Data are expressed as the mean ± standard deviation. HIV, HSV-stimulated, HSV-immune splenocytes; HI, HSV-immune splenocytes; FIV, influenza virus-stimulated, influenza virus-immune splenocytes; HIV CY, splenocytes from HSV-immune mice pretreated with cyclophosphamide (50 mg/kg) 48 h prior to sacrifice.

^b —, Untreated controls for each CTL induction culture tested.

^c Significantly different from the untreated controls at $P < 0.01$.

wise, supernatants from HSV-stimulated normal splenocytes did not contain the suppressor factor, thus indicating that infection with the virus was an insufficient stimulus for suppressor induction. Finally, the presence of I-J determinants also makes unlikely the possibility that the suppressor molecule was of viral origin. Another possibility is that a host-derived factor directly bound a viral protein. However, we suggest that unless the suppressor factor covalently bound the viral antigen (which is a remote possibility), such a complex would not be intact following elution from the anti-I-J immunoaffinity column; this would result in multiple bands being detected on silver-stained HSV-SF. Multiple bands were observed, but the single band which was visualized on both the anti-HSV and anti-I-J Western blots was of the same relative molecular weight, whereas no other bands reacted with the polyclonal anti-HSV antiserum. Therefore, it is unlikely that HSV-SF is an I-J⁺ factor which binds antigen, because bands which reacted only with the anti-HSV antiserum and not with the anti-I-J antiserum were not observed. In contrast, antigen-specific I-J⁺ suppressor factors which express anti-idiotypic determinants have been demonstrated in other systems (13, 50, 53). Such factors play an important role in the transmission of suppressogenic signals in hapten-specific suppressor cell circuits (12, 17, 18, 22). A characteristic of these factors is that they are frequently composed of two subunits held together by disulfide bonds (3, 15, 31), although single-chain factors have also been described (24, 26). At this time, it is uncertain whether the suppressor factor described in this communication is of the one- or two-polypeptide-chain variety. This topic is still under investigation.

At this time we can only speculate on how the HSV-specific suppressor factor (HSV-SF) may be involved in the regulation of HSV-specific CTL and lymphoproliferative responses. By analogy with hapten systems, we suppose that the factor forms part of a regulatory cell circuit that modulates immune responsiveness to HSV. In support of this, elsewhere we have demonstrated the involvement of at least three cell types and two factors in the circuit that controls HSV-specific CTL and lymphoproliferative responses *in vitro* (21, 22). As such, we consider HSV-SF to be a product of a second-order suppressor (Ts₂) cell which we have identified as a Lyt 2⁺, I-J⁺, HSV-immune T lymphocyte, presumably anti-idiotypic in its antigenic specificity. As observed in hapten systems (2, 12, 13), Ts₂ cells produce an anti-idiotypic suppressor factor which is also I-J⁺. This antigen-specific factor fails to mediate suppression directly. Instead, it is recognized by an antigen-activated acceptor (Ts₃) cell that responds by producing nonspecific suppressor factors that apparently act as the actual suppressors (34). Our results showed that spleen cells from HSV-immune mice generated nonspecific suppressor activity when exposed to HSV-SF along with antigen. The production of the nonspecific suppressor activity was shown to require cyclophosphamide-sensitive, HSV-primed splenocytes which expressed the Lyt 1⁺2⁻, I-J⁺ phenotype. Accordingly, the cell resembles the suppressor-acceptor cell described by Asherson et al. that regulates hapten-specific contact sensitivity (2, 34). However, in the picryl chloride model, the cyclophosphamide-sensitive, suppressor-acceptor cell was identified as a Lyt 2⁺ cell and was present in mice which were contact sensitized, although not necessarily with picryl chloride. Thus, the system we describe is similar although not identical to the hapten model. However, final comparisons must await the development of more purified cell populations and suppressor T-cell hybridomas.

It is important to understand how suppressor cells and factors interact with the immune system *in vitro*, since clues which will prove of value *in vivo* may emerge on how to manipulate the system. For example, suppressor cells could serve to inhibit the protective aspects of immunity prior to the development of recrudescence. There is some evidence that helper/suppressor T-lymphocyte ratios do change around the time of recrudescence in humans (48); recently, it has been shown in the guinea pig model of HSV type 2 recrudescence that suppressor cells are present in the spleen at the time of recrudescence (23). If suppressor cells and their products are indeed involved in modulating immunity to HSV in humans, they could provide a useful target for treatment aimed at breaking the cycle. Whether the operation of a similar suppressor cell circuit accounts for the variation of host responsiveness that accompanies the development of herpesvirus recrudescence disease requires further investigation.

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