JOURNAL OF VIROLOGY, June 1985, p. 798-803
0022-538X/85/060798-06$02.00/0
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Vol. 54, No. 3

Production of Soluble Suppressor Factors by Herpes Simplex Virus-Stimulated Splenocytes from Herpes Simplex Virus-Immune Mice

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Received 15 October 1984/Accepted 1 March 1985

Indirect evidence indicates that herpes simplex virus (HSV)-specific cytotoxic-T-lymphocyte induction is regulated by suppressor cells. To search for such suppressor effects, supernatant fluids from splenocyte cultures from normal and HSV-immune mice cultured either with or without viral stimulation were tested for their ability to inhibit HSV-specific cytotoxic-T-lymphocyte induction. Only the supernatant fluid from the HSV-stimulated, HSV-immune cultures contained a suppressor activity (HSV-SF). HSV-SF was produced by nylon-wool-purified Thy 1^+ cells. HSV-SF was detectable after 3 days of culture and would only suppress cytotoxic-T-lymphocyte induction if HSV-SF was added within 24 h of initiation of the test cultures. HSV-SF was neither dialyzable nor heat stable. Molecular sieve chromatography of HSV-SF yielded multiple peaks of suppressor activity. Although most of these peaks exhibited nonspecific suppressor activity, the suppression mediated by the 90,000 to 150,000-molecular-weight fractions was antigen specific and genetically restricted. These results provide direct evidence for the regulation of HSV-cytotoxic-T-lymphocyte induction by a novel suppressor factor.

Cell-mediated immunity plays a critical role in recovery from herpes simplex virus type 1 (HSV) infections (17), and several models have been described for identifying specific cell-mediated immunity responses to HSV. Antiherpetic activity by macrophages (21), natural killer cells (12), delayed-type-hypersensitivity-mediating T cells (14), and cytotoxic T lymphocytes (CTL) (11) has been described. Of these, HSV-specific CTL activity has proven the most difficult to demonstrate. Either pretreatment with cyclophosphamide or in vitro cultivation is necessary to demonstrate HSV-specific CTL activity in lymph nodes (16) and spleens (11) of infected animals. Therefore, it has been suggested that HSV-specific CTL induction is regulated by a suppressor cell which is inactivated by either in vitro cultivation or low-dose cyclophosphamide treatment (11, 16). Although limiting dilution analysis of HSV-specific CTL precursors has provided additional indirect evidence of suppression (18), no direct evidence of HSV-specific CTL suppression has been forthcoming despite reports of suppressor cells and factors regulating HSV-specific delayed-type hypersensitivity (15) and lymphoproliferative responses (8).

In this communication suppressor factors are described which regulate the in vitro induction of HSV-specific CTL. HSV-immune splenocytes stimulated with HSV produced suppressive supernatant fluids which when fractionated on a gel filtration column contained multiple suppressor activities. One of these fractions exhibited both HSV-specific and genetically restricted activity.

MATERIALS AND METHODS

Viruses, cells, and mice. HSV strain KOS was propagated in HEp-2 cells by the method of Bone and Courtney (4). Viral stocks used had an infectivity titer of 4×10^8 PFU/ ml. The virus was UV inactivated by exposing 0.5 ml of the viral stock to a germicidal lamp (Sylvania Electric Products) at a distance from the lamp of 3 cm for 2 min. UV inactivation resulted in a reduction of viral titer to fewer than 10^2 PFU/ml. Influenza virus A/Puerto Rico/8/34 was obtained from J. Bennick, Wistar Institute, Philadelphia, Pa., and propagated in chicken eggs. Influenza virus stock had an infectivity titer of 1,200 hemagglutination units per ml. 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 Div., Oak Ridge National Laboratory, Oak Ridge, Tenn., and EL4 thymoma cells (H-2^b) were obtained from Mary Hilfiker, Cleveland Clinic Foundation, Cleveland, Ohio. The cells were cultured in McCoys 5A 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. (9). C3H/HeJ mice (H-2^k) were obtained from the breeding colony at the University of Tennessee Memorial Research Center Hospital, Knoxville, Tenn. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from Cumberland View Farms, Clinton, Tenn.

Mouse immunizations and preparation of splenocyte cultures. Mice (4 to 6 weeks old) were injected intraperitoneally 4 weeks before use with 0.1-ml inocula containing either 10^6 PFU of HSV or 40 hemagglutination units of influenza. Single-cell splenocyte suspensions were prepared as described elsewhere (11). Splenocytes were adjusted to 4×10^6 cells per ml in RPMI 1640 containing 5% heat-inactivated fetal calf serum (GIBCO Laboratories), 7 mM glutamine, penicillin (100 μ/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and 5×10^{-5} M 2-mercaptoethanol. For bulk cultures 10 ml of the cell suspension was added to 100-mm culture plates (Corning Glass Works, Corning, N.Y.), and for microcultures 0.1 ml of the suspension was added to each well of a flat-bottomed, 96-well plate (Corning). HSV (multiplicity of infection was 1.0 before UV inactivation) or influenza (100 hemagglutination units) was added to each culture in complete medium to a final volume of 15 or 0.2 ml for bulk cultures or microcultures, respectively.

Cell separation. Splenocytes were separated into various subpopulations by differential adherence and antibody-plus-

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TABLE 1. Inhibition of HSV-specific CTL (H-2^k) induction by supernatant fluids from HSV stimulated, HSV-immune splenocyte cultures[a]

Supernatant tested[b]	% Specific ^{51}Cr release[c]	
	LHSV (H-2^k)	EHSV (H-2^b)
None	44.2	14.2
Normal	42.4	13.9
Normal and HSV	46.5	12.5
Immune	38.5	10.1
Immune and HSV	5.8	2.4
Immune and HSV; 60°C, 15 min	41.5	14.9

[a] HSV-specific CTL induction cultures consisted of 4×10^5 HSV-immune splenocytes incubated with HSV (multiplicity of infection, 1.0) in 200-μl volumes in 96-well flat-bottomed plates for 5 days. On day 5 100 μl of cells from each well was transferred to a 96-well round-bottomed plate, and 10^4 HSV-infected, ^{51}Cr-labeled syngeneic (LHSV) or allogeneic (EHSV) targets were added in a 100-μl volume. Then the plates were centrifuged and incubated for a 3-h ^{51}Cr-release assay.

[b] HSV-immune and normal splenocytes were incubated with or without HSV stimulation for 5 days. In some experiments the splenocytes were treated with anti-Thy 1.1 antiserum and complement before incubation with HSV. The culture supernatant then was harvested, dialyzed, and filter sterilized before being added to the HSV-specific CTL induction culture (20% [vol/vol]). In some cases a portion of the supernatant from HSV-stimulated, HSV-immune splenocyte cultures was heated at 60°C for 15 min before dialysis.

[c] % Specific ^{51}Cr release [(experimental − spontaneous)/ (total − spontaneous)] × 100. The standard error of the mean did not exceed 10%.

complement (C') depletion. Nylon-wool-purified cells were prepared by adhering 10^7 splenocytes to 0.5 mg of nylon wool in a 10-ml disposable syringe (11). The nonadherent cells were eluted with 15 ml of prewarmed RPMI 1640. The yield from the column was approximately 35%, and the eluted cells were >99% Thy 1^+, immunoglobulin negative (data not shown). Antibody and complement depletion was performed as detailed elsewhere (11). Briefly 10^7 cells were incubated with 1.0 ml of anti-Thy 1.2 antiserum (Cedarlane Laboratories) for 45 min on ice. The cells were washed once with cytotoxicity medium (Cedarlane) and then resuspended in 1.0 ml of rabbit C (Cedarlane). After 30 min of incubation, the cells were rinsed several times with cytotoxicity medium and then resuspended in fresh RPMI 1640 containing 5% fetal calf serum.

Preparation of culture supernatant fluids. Supernatant fluids from HSV-immune cultures were collected at various intervals after initiation of the bulk culture. The cells were pelleted by centrifugation (500 × g for 15 min) and discarded. The supernatant fluid was dialyzed overnight against RPMI 1640, filter sterilized (0.2 μM Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.), and stored frozen at −70°C. Serum-free 5-day supernatants for biochemical fractionations were prepared by incubating the splenocytes for 3 days in complete medium, pelleting the cells, and then suspending the cells in serum-free RPMI 1640. After an additional 48-h of incubation, the serum-free conditioned medium was concentrated by ultrafiltration on an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.) and dialyzed overnight against phosphate-buffered saline (pH 7.2) containing 0.05% polyethylene glycol (3350; Sigma Chemical Co., St. Louis, Mo.).

Assay of culture supernatant fluids. Supernatant fluids were assayed for suppressive activity by adding them at initiation to HSV-stimulated, HSV-immune splenocyte microcultures. In some experiments, the supernatant was added at various intervals after culture initiation. Suppressive supernatant fluids were routinely assayed at a final concentration of 20% (vol/vol). In some experiments, supernatants were assayed at concentrations ranging from 1 to 50% (vol/vol). The test culture was harvested on day 5, and the cytotoxic activity of the wells was determined.

Cytotoxicity assay. HSV-infected syngeneic and allogeneic targets were prepared as previously described (11). Influenza-infected targets were prepared in serum-free media. The cells were incubated with the virus at least 2 h before 100 μCi of $Na_2{}^{51}CrO_4$ (ICN Pharmaceuticals, Inc., Irvine, Calif.) was added. The cells then were incubated for 2 h at 37°C and washed several times with medium. The ^{51}Cr-labeled targets were suspended at 10^5 cells per ml.

Splenocytes in the microcultures were harvested on day 5 for the determination of cytotoxic activity. A 0.1-ml volume from each well was transferred to two separate round-bottomed microtiter plates. The cells were pelleted, the supernatant fluid was discarded, and the cell pellet was suspended in 0.1 ml of RPMI 1640. Target cells were added to each well in 0.1-ml volumes containing either 1×10^4 or 2×10^3 target cells. This resulted in a ratio of effector to target cells of approximately 12:1 or 60:1, respectively (data not shown). The results presented in this manuscript are at the lower ratio of effector to target cells unless otherwise indicated. The microtiter plates were centrifuged at 200 × g for 1 min before incubation for 3 h at 37°C. After incubation 0.1 ml of supernatant from each well was harvested for measurement of radioactivity, reported as percent specific ^{51}Cr release [(effector cell release − medium control release)/(detergent release − medium control release)] × 100. Results reported are the average values obtained at 10^4 target cells per well unless otherwise indicated.

Gel filtration chromatography of culture supernatant fluids. Gel filtration chromatography was performed with Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, N.J.). Column dimensions were 100 by 1.5 cm, and the gel was equilibrated with phosphate-buffered saline (pH 7.2) containing 0.05% polyethylene glycol 3350 at 4°C. The column was calibrated with protein standards with molecular weights of 150,000, 65,000, and 12,000. A 1-ml volume of concentrated (100:1) serum-free suppressive supernatant was loaded on the column and eluted with phosphate-buffered saline containing polyethylene glycol. Fractions were collected, filtered, and assayed at 0.1 dilution by the microculture method.

Statistical analysis. The reported results are representative of experiments performed at least three times. The values reported are the mean determination from four replicates. Student's *t* test was used to analyze the data.

RESULTS

Production of a soluble suppressor activity by stimulated HSV-immune splenocytes. Incubation of HSV-immune splenocytes with HSV resulted in the induction of HSV-specific secondary CTL (Table 1). The supernatant fluids from these cultures also contained a nondialyzable heat-labile suppressor activity for HSV-specific CTL induction (HSV-SF). However, production of the suppressor activity did not always correlate with CTL induction as CTL-negative cultures did produce HSV-SF (data not shown). Production of this suppressor activity increased throughout the culture period (Fig. 1); however, statistically significant ($P < 0.01$) levels of suppressor activity were noted only with HSV-SF obtained after 3 days of incubation. The supernatant fluids from normal, HSV-stimulated normal, HSV-stimulated influenza-immune, or unstimulated immune splenocytes were neither suppressive nor enhancing (data not shown).

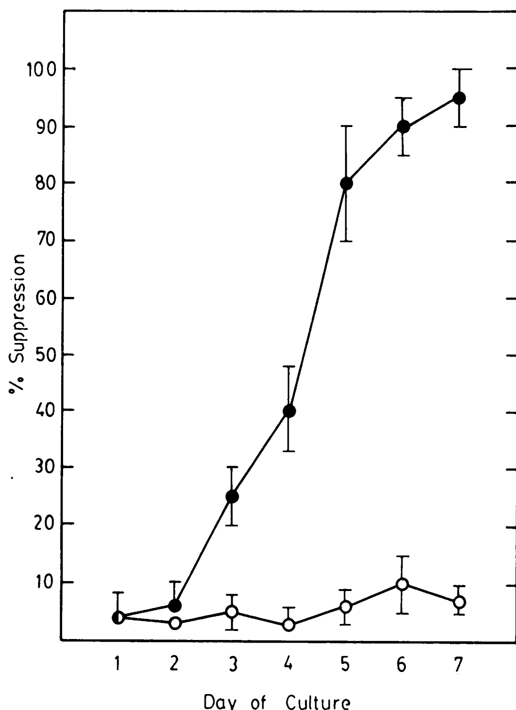

FIG. 1. Time course of the production of suppressor activity. Supernatant fluid from HSV-immune splenocytes incubated with (●) or without (○) HSV stimulation was collected at daily intervals and later assayed for suppressor activity against HSV-specific CTL induction. Each point represents the mean (± standard deviation) for four replicates.

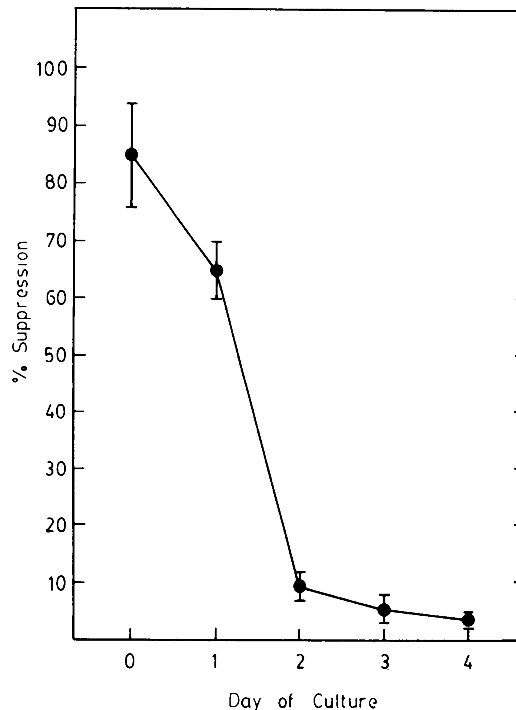

FIG. 2. Time course of suppressor activity. A suppressive supernatant from HSV-stimulated, HSV-immune splenocyte cultures was added to HSV-specific CTL induction cultures at daily intervals after culture initiation. Each point represents the mean (± standard deviation) for four replicates.

Identification of the cell population responsible for HSV-SF production. HSV-immune splenocytes were incubated for 3 days with HSV antigens and then separated into different cell populations. The separated cells then were cultured for an additional 3 days, and their supernatant fluids were collectedand assayed for HSV-SF activity. The nylon-wool-purified cell population produced HSV-SF as did the plastic nonadherent cells (Table 2). Treatment with anti-Thy 1.2 antiserum and complement at day 0, followed by virus stimulation, also abrogated HSV-SF production. Together these results indicate that the production of HSV-SF requires T-cell participation.

TABLE 2. T-cell requirement for production of HSV-induced suppressor factors from HSV-immune splenocytes[a]

Treatment[b]	% Suppressor activity of supernatant[c]
None	100
Nylon wool nonadherent	115
Plastic adherent	0
Plastic nonadherent	95
Anti-Thy 1.2 + C′	5
Anti-immunoglobulin + C′	105

[a] HSV-immune splenocytes were incubated for 3 days with HSV antigens in bulk cultures and then separated into various subpopulations based on adherency to plastic and nylon wool. The cells were incubated for an additional 3 days in fresh media. The supernatants from these cultures were harvested and assayed for HSV-SF activity as described in Table 1, footnote *a*. In the case of antibody and C treatments, splenocytes were treated on day 0 before antigen stimulation. The supernatants were harvested on day 6.

[b] The treatment procedures are described in detail in the text. Plastic adherent cells were those cells which remained on the culture dishes after the dishes were rinsed with warm media on day 3. Plastic nonadherent cells were those collected by rinsing the plates with warm media.

[c] % Suppressor activity of supernates (Experimental/control) × 100%. The no-treatment control (None) reduced CTL activity in the treated culture from 48.4% specific lysis to 10.2%, a reduction of 79%. This value was used as the control value in the above formula to determine % suppressor activity of the supernatants.

Time course of suppression by the stimulated immune splenocyte supernatant fluids. In the preceding experiments, the HSV-SF was added at the initiation of the test cultures. In experiments in which HSV-SF was added to the test culture at different times, maximum suppression of CTL induction was observed only when HSV-SF was added during the first 24 h of incubation (Fig. 2). This indicated either that the target of the suppressor factor was an early event in HSV-specific CTL induction or that the suppressive activity represented an afferent acting suppressor factor.

Genetic restriction of the activity of the suppressive supernatants. A characteristic in other systems of afferent acting suppressor factors is that the activity is genetically restricted (2). To test for this in the present system, the HSV-SF generated by C3H/HeJ (H-2^k)-stimulated immune splenocytes was assayed against C57BL/6 (H-2^b) and BALB/C (H-2^d) cultures. The results (Table 2) indicate that although HSV-SF sometimes exhibited genetic restriction in its activity (Table 3, experiment 1), variations in the genetic restrictiveness were observed among batches of HSV-SF, implying the possibility of multiple suppressive factors. To separate the genetically restricted activity from nonrestricted activity, serum-free HSV-SF was fractionated on an S-200 gel filtration column. When the column fractions were assayed against syngeneic and allogeneic HSV-specific CTL induction and syngeneic influenza-specific CTL induction (Fig. 3),

TABLE 3. MHC-restriction of suppressive supernatant[a]

Expt	Strain tested	H-2	Supernatant added[b]	MHC restriction of[c]: L HSV ($h-2^k$)	E HSV ($H-2^b$)	A HSV ($H-2^d$)
1	C3H/HeJ	k	−	35.7	15.0	ND
	C3H/HeJ	k	+ (A)	5.2	1.7	ND
	C57BL/6	b	−	1.3	33.0	ND
	C57BL/6	b	+ (A)	5.4	29.4	ND
2	C3H/HeJ	k	−	38.4	13.5	ND
	C3H/HeJ	k	+ (B)	3.2	0.5	ND
	C57BL/6	b	−	3.0	39.7	ND
	C57BL/6	b	+ (B)	2.5	17.7	ND
3	C3H/HeJ	k	−	48.7	ND	11.4
	C3H/HeJ	k	+ (C)	11.9	ND	3.3
	C3H/HeJ	k	+ (D)	2.5	ND	0
	BALB/c	d	−	4.3	ND	33.7
	BALB/c	d	+ (C)	1.7	ND	5.9
	BALB/c	d	+ (D)	4.0	ND	34.0

[a] A suppressive supernatant was added at 20% (vol/vol) concentration to HSV-stimulated immune splenocyte cultures at initiation. CTL activity against syngeneic and allogeneic targets was assayed on day 5 as described in Table 1, footnote *a*.

[b] The letter within parentheses indicates a different batch of C3H/HeJ ($H-2^k$)-generated suppressive supernatant used for a given experiment.

[c] ND, Not done. Data represent the average of four replicates per experiment. Standard errors were routinely less than 5% of the mean values. The L targets were L929 cells infected with HSV-1 (L HSV). The E targets were EL4 thymoma cells infected with HSV-1 (E HSV). The A targets were BALB/c 3T3 clone A31 cells infected with HSV-1 (A HSV).

several distinct peaks of nonspecific suppressor activity were observed. In addition, antigen-specific, genetically restricted suppressor activities were observed in the fractions corresponding to an apparent molecular weight of 90,000 to 150,000. When those fractions containing the antigen-specific major histocompatibility complex (MHC)-restricted suppressor activity were concentrated, rechromatographed on an S-200 gel, and assayed against C3H/HeJ HSV-specific CTL induction cultures, a single peak of activity at a molecular weight of 90,000 to 150,000 was observed. No suppressive activity was observed against either C57BL/6 HSV-specific CTL cultures or C3H/HeJ influenza-specific CTL cultures (Fig. 4), confirming the suggested presence of an antigen-specific, genetically restricted suppressor factor in HSV-SF.

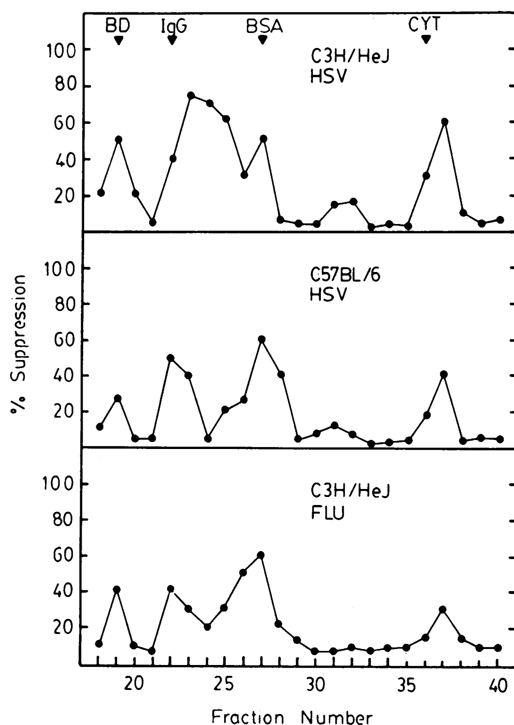

FIG. 3. S-200 fractionation of serum-free suppressive supernatant. A suppressive supernatant produced by C3H/HeJ HSV-immune splenocytes stimulated with HSV was chromatographed on a Sephacryl S-200 gel filtration column. The eluted fractions were assayed for suppressor activity against C3H/HeJ HSV-specific CTL, C57BL/6 HSV-specific CTL, and C3H/HeJ influenza (C3H/HeJ-FLU)-specific CTL induction cultures for evidence of genetic restriction and antigenic specificity of the suppressor activities. Specific ^{51}Cr releases for the cultures were 43.3, 34.7, and 24.2% for C3H/HeJ-HSV, C57BL/6-HSV, and C3H/HeJ-FLU, respectively.

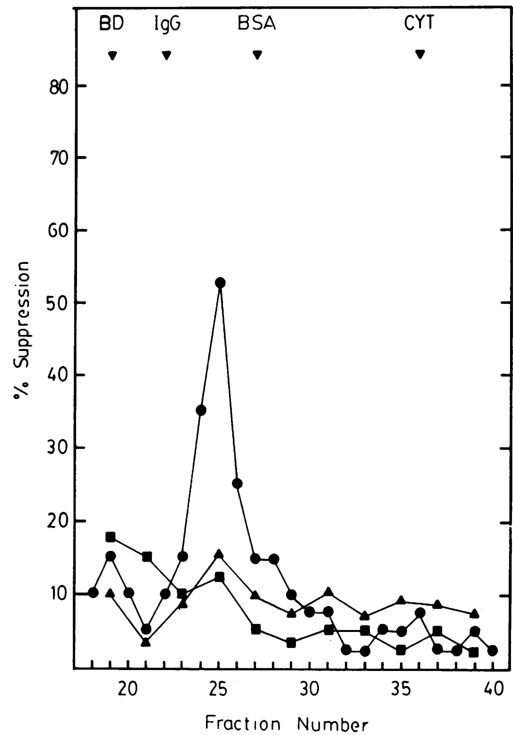

FIG. 4. Rechromatography of genetically-restricted, antigen-specific suppressor activity. Fractions 24, 25, and 26 from the S-200 column (Fig. 3) were rechromatographed on the S-200 column. The eluted fractions were again assayed against C3H/HeJ HSV-specific CTL (●), C57BL/6 HSV-specific CTL (■), and C3H/HeJ influenza-specific CTL (▲) induction cultures. Specific ^{51}Cr releases were similar to those reported in Fig. 3.

DISCUSSION

Suppressor cells (1, 13, 20, 22) and soluble factors (3, 6, 10, 23) are known to regulate the induction of alloantigen-specific CTL, but the role such regulatory elements play in virus-specific CTL induction is unknown. Previous evidence that HSV-specific CTL induction is regulated by a suppres-

sor mechanism was indirect (11, 16, 18). In the present report we provide direct evidence that induction of in vitro HSV-specific CTL is regulated by soluble suppressor factors produced by HSV-specific T lymphocytes (HSV-SF). HSV-SF were generated in cultures of HSV-immune splenocytes after viral stimulation. Similar factors were not produced by HSV-stimulated normal, HSV-stimulated influenza-immune, or unstimulated HSV-immune splenocytes, indicating that activation by specific antigen is required for both in vivo priming and in vitro activation to generate suppressor factors. From cell separation studies, T lymphocytes were shown necessary for HSV-SF production, but the role of other interacting cell types and the T-cell subset nature of producing cells require further investigation. Cultures which produced suppressor factors usually generated CTL, indicating that factor production failed to abrogate CTL induction in the factor-producing culture. Presumably this was because factor production occurred too late to regulate CTL. Thus HSV-SF levels were first detectable on day 3 and did not peak until day 5. To effect suppression and abrogate CTL, induction factors had to be added within 24 h of the initiation of culture.

Some batches of HSV-SF exhibited genetically restricted suppressor activity, whereas others inhibited CTL induction in a nonspecific fashion. These results indicated that HSV-SF might be heterogenous in composition. Support for this notion came from results of fractionation of HSV-SF by molecular sieve chromatography. Multiple peaks of suppressor activity were observed. Most of those peaks exhibited nonspecific activity, but the suppression mediated by the 90,000 to 150,000-molecular-weight fractions was antigen specific and genetically restricted.

Although antigen-specific regulation of CTL induction by suppressor cells was described previously (1, 20), regulation by soluble factors was not antigen specific (3, 10, 23). Thus the antigen-specific, genetically restricted suppressor factor regulating HSV-specific CTL induction that we observed represents a novel mechanism for CTL regulation. Antigen-specific, genetically restricted suppressor factors are involved in the regulation of antibody production, contact sensitivity, and delayed-type-hypersensitivity reactions, and similar factors act as suppressor signals between various subpopulations of T cells that comprise antigen-specific suppressor networks (reviewed in references 5 and 7). Our finding of an antigen-specific, genetically restricted suppressor factor in HSV-SF indicates that a similar suppressor network may be involved in the regulation of HSV-specific CTL induction. Furthermore, the antigen-specific suppressor networks were recently shown to culminate in the production of a nonspecific suppressor factor that mediates suppression (2, 24). It is possible that the nonspecific factors described in this report may be part of the same suppressor network.

It is important to ascertain the mode of action of the specific and nonspecific suppressor factors. However, from analogy with alloantigen systems, we suspect that the target cells could be helper T lymphocytes known to be required for HSV-specific CTL induction (2, 19). Thus the necessity to add factors within 24 h to obtain suppression also was observed in the alloantigen system (10, 23). In the latter instance, helper T cells were identified as targets on the basis of cell separation analysis and because suppression could be overcome by the addition of helper cell products and cells (10, 22). In our system we were able to inhibit some but not all of the suppressor activities by the addition of exogenous sources of IL-2-containing supernatant fluids from mitogen-stimulated splenocyte cultures (data not shown). These results indicate that more than one mechanism of suppression may be operative in addition to possible effects on helper cell activity. Experiments to fully elucidate the nature of suppression currently are underway.

Although our work demonstrates that CTL induction in vitro can be regulated by suppressor factors, the in vivo significance of such factors remains to be demonstrated. Others have proposed that such factors may be involved in recrudescent disease (8). When the guinea pig model for herpes simplex virus type 2 recrudescence was used, virus reactivation in vivo was shown to result in the generation of suppressor cells which produce suppressor factors upon in vitro incubation. Iwasaka et al. suppose that similar factors could suppress protective aspects of immunity, thus permitting sufficient virus spread to cause lesions. However, whether such factors are produced in vivo or whether in vitro-generated factors can modulate immunity in vivo has not been shown. We are currently attempting to develop direct assays for the detection of factors in vivo which do not require in vitro cultivation.

ACKNOWLEDGMENTS

The technical assistance of Linda Miller was greatly appreciated.

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