JOURNAL OF VIROLOGY, Apr. 1984, p. 56–59
0022-538X/84/040056-04$02.00/0

Vol. 50, No. 1

Role of T-Lymphocyte Subsets in Recovery from Herpes Simplex Virus Infection

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Received 12 September 1983/Accepted 16 December 1983

Our investigations probed the nature of different T-lymphocyte subsets effecting clearance of herpes simplex virus after infection of the pinna. Cell populations from animals recently infected subcutaneously or intraperitoneally (acute population) or from animals infected 6 weeks previously (primed population) or the latter cells reimmunized in vitro with virus (memory population) were studied. Viral clearance was a function of the Lyt 1^+2^- subset in the acute population, but with the memory population both Lyt 1^+ and Lyt 2^+ cells effected clearance. In primed populations, viral clearance was effected only by the Lyt 2^+ subset. The ability of the various cell populations to adoptively transfer delayed-type hypersensitivity was also studied. Only acute population cells from animals infected subcutaneously and memory population cells transferred delayed-type hypersensitivity. In both cases, the cell subtype was Lyt 1^+2^-. Our results demonstrated that the delayed-type hypersensitivity response does not always correlate with immunity to herpes simplex virus. Multiple subsets of T cells participate in viral clearance, and their respective importances vary according to the stage of the virus-host interaction.

The role of different components of natural and acquired immunity in controlling herpesvirus infections remains unresolved, although the T-lymphocyte system appears to play the principal role in recovery from infection (1, 4, 13, 16; B. T. Rouse *in* B. T. Rouse and C. Lopez, ed., *Immunobiology of Herpes Simplex Virus Infection*, in press.) However, the respective importance of different subsets of T cells is unclear. Based on the observation that mice infected intravenously with herpes simplex virus (HSV) fail to generate delayed-type hypersensitivity (DTH) responses yet can still mount other forms of T-lymphocyte activity and remain competent to clear virus (11), it appears that the DTH response is not essential for immunity. In contrast, studies on adoptive cell transfers have indicated that the class II major histocompatibility complex-restricted DTH-mediated T lymphocytes play the dominant role in protection from lethality (4) as well as in clearance of virus from the site of infection (8, 10). Thus, cells from donor mice tolerant with respect to DTH were less protective and failed to clear virus from the ear. Furthermore, at least two groups claim that adoptive transfer of immunity is an exclusive property of the class II major histocompatibility complex-restricted Lyt 1^+ T lymphocyte (8, 10), which is the only cell type that mediates DTH against HSV (12).

The purpose of the present investigation was to reassess the role of Lyt 2^+ HSV-specific T lymphocytes in clearance of virus from the site of local infection. Our results demonstrate that such cells can function this way and that under certain conditions may provide the principal means of viral clearance.

MATERIALS AND METHODS

Cells and viruses used. Strain L929 cells ($H\text{-}2^k$) were obtained from Flow Laboratories, Inc., McLean, Va., and BALB/c 3T3 clone A31 cells ($H\text{-}2^d$) were obtained from Ray Tennant, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. L929 cells and A31 cells were both grown in McCoy 5A medium supplemented with 5% newborn calf serum. HSV type 1 (HSV-1) strain KOS was propagated in HEp-2 cells as described by Bone and Courtney (2). The viral stocks used had infectivity titers ranging from 1×10^8 to 3×10^8 PFU/ml.

Cyclophosphamide treatment and inoculation of virus. Mice were injected intraperitoneally (i.p.) with 60 mg of cyclophosphamide per kg 24 and 48 h before virus inoculation. HSV-1 (2×10^6 PFU in 20 μl) was inoculated into the pinna of the right ear of anesthetized mice 2 h before the adoptive transfer of cells.

Mouse immunization and preparation of splenocytes. C3H/HeJ mice were obtained from the University of Tennessee Medical Research Center. Mice 6 to 9 weeks of age were used as recipients in the adoptive transfer studies. Animals serving as cell donors were infected subcutaneously (s.c.) or i.p. with 0.1 ml of inoculum containing 10^6 PFU of infectious HSV-1 KOS. At least 6 weeks after i.p. infection, the mice were killed by cervical dislocation, and their spleens were removed aseptically for preparation of single-cell suspensions as described elsewhere (6). In those cases where transfer of restimulated cells was desired, spleen cells were adjusted to 2×10^6/ml in RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, penicillin (100 μ/ml), streptomycin (100 μg/ml), and 5×10^{-5} M 2-mercaptoethanol and were added to plastic tissue culture plates at a density of 10^6 cells per cm^2 of surface area. UV light-inactivated virus (10^7 PFU) was subsequently added, and the spleen cell cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 5 days, the cells were harvested and washed in medium, and the cell density was adjusted to the desired concentration.

Cytotoxicity assay. The assay for cytotoxic T lymphocytes (CTL) was carried out as previously described (6). Splenocytes were cultured for 5 days in vitro with 10^7 PFU of UV-irradiated HSV-1 to induce secondary CTL. Cells were assayed with syngeneic and allogeneic virus-infected and uninfected target cells at effector-to-target-cell ratios of 25:1 and 5:1, and the results were reported as percent specific lysis (as described in detail previously [6]).

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TABLE 1. Adoptive transfer of DTH by immune spleen cells[a]

No. of cells transferred[b]	Avg % increase in footpad thickness over the control			
	7-day immune (s.c.)	7-day immune (i.p.)	6-wk immune (i.p.)	Virus-stimulated 6-wk immune (i.p.)
5×10^6	27 ± 2*	8 ± 1	3	36 ± 4*
5×10^5	9 ± 1	3	0	26 ± 2*
1×10^5	5	0	0	18 ± 2*
5×10^4	0	0	0	12 ± 1
1×10^4	0	0	0	10 ± 1

[a] Cell transfers were given intravenously, and 2 h later the eight mice in each group were challenged in the right footpad with virus and in the left footpad with HBSS (control). Footpad thicknesses were measured 24 h later. Values with an asterisk were significantly greater than those for control feet given HBSS. All mice received 10^6 PFU of virus by either the s.c. or i.p. route.

[b] Splenocytes were stimulated in vitro for 5 days with UV-inactivated HSV.

Cell fractionation. Splenocytes infected 1 week previously were harvested and washed twice, and the viable cells were isolated by centrifugation with Lympholyte-M density separation medium (Accurate Chemical and Scientific Corp., Westbury, N.Y.). The banded cells, or those treated with antisera plus complement to remove selected cell populations, were adoptively transferred into infected recipients. Antibody treatment was accomplished by reacting 100×10^6 spleen cells with 7 ml of monoclonal anti-mouse Lyt 1.1, Lyt 2.1, or Thy 1.2 diluted 1;30 (Accurate) for 1 h at 4°C with occasional mixing. The cells were then washed, and 7 ml of a 1:8 dilution of Low-Tox-M rabbit complement (Accurate) was added. The mixture was incubated for 1 h at 37°C with occasional mixing. After complement treatment, the cells were washed and adjusted to appropriate numbers for injection.

Infectivity assay. At 72 h after cell transfer the pinnae from the mice were removed and homogenized in 1 ml of RPMI–10% fetal calf serum. The suspensions were then diluted for independent assay, and 0.2 ml of each dilution was plated into microwells containing confluent monolayers of Vero cells. Each well was read for plaques 72 h later.

Measurement of DTH. The protocol employed for the measurement of DTH was a modification of the method reported by Crowle (3). In brief, mice were inoculated with 5×10^6 PFU (25 μl) of virus in the right footpad and 25 μl of Hanks balanced salt solution (HBSS) in the left hind footpad. Control mice received HBSS in both footpads. The thickness of the footpads was measured with calipers 24 h after challenge. The DTH response was expressed as the average percent footpad increase in pads given virus versus pads given HBSS. Significance was determined by the Student t test at 95% confidence limits.

Adoptive transfer of DTH. Cells in 200 μl of HBSS were injected intravenously into groups of three mice, and approximately 60 min later HSV was injected into the right footpad and HBSS into the left. Swellings were measured at 24 h after injection.

RESULTS

Adoptive transfer of DTH by secondary HSV immune splenocytes. In confirmation of the reports of others (9, 14), the adoptive transfer of splenocytes from mice 7 days after s.c. injection with HSV to syngeneic recipients conferred the DTH response (Table 1). In addition, if donor mice were infected with virus via the i.p. route, the DTH response transferred was either weak or undetectable. When splenocytes from mice infected i.p. 6 weeks previously were secondarily stimulated in vitro for 5 days with virus (memory population), the cell population generated high levels of *H-2*-restricted cytotoxicity and also became competent at transferring DTH (Table 1). In fact, the memory population was around 10-fold more effective at mediating DTH than were spleen cells from animals infected s.c. 7 days previously. In all cases, mice infected i.p. 6 weeks previously failed to give DTH responses, and their splenocytes were incapable of transferring DTH before secondary stimulation. These results could mean that in vitro culture either expands the Lyt 1^+ HSV-specific cells or abrogates the effect of any suppressor cell.

As is the case with lymphoid populations from animals recently infected in vivo (8, 10), the cell type responsible for DTH transfer in the memory population was the Lyt 1^+2^- subset. Thus, even Lyt 2^+ cell populations with potent HSV-specific cytotoxic activity were incapable of mediating DTH (Table 2).

Viral clearance from the pinna. Previous reports from the Nash group had provided strong evidence that clearance of HSV from the local site of infection was an exclusive property of T lymphocytes of the Lyt 1^+2^- subset (10). With 7-day splenocytes, we also found that the cell type principally involved in viral clearance was of the Lyt 1^+2^- subset (Table 3). With the memory population, however, Lyt 2^+ cells as well as Lyt 1^+ cells could mediate viral clearance in the majority of experiments (Table 4). Of the two T-lymphocyte subsets, the efficacy of the Lyt 1^+ population was the greater. In an experiment in which the clearance activities of various numbers of Lyt 1^+ and Lyt 2^+ cells were compared, the former were at least 10-fold more effective than Lyt 2^+ cells at clearance (Table 5). The culture of normal cells for 5 days with viral antigen failed to generate cells capable of mediating DTH or viral clearance (data not shown), thus indicating that primary T-cell responses to HSV were not generated in vitro.

TABLE 2. Effect of T-cell subset depletion on the adoptive transfer of DTH

Cell treatment[a]	Expt no.			
	1		2	
	Avg % footpad increase[b]	% Specific lysis[c]	Avg % footpad increase[b]	% Specific lysis[c]
Complement	45 ± 6*	43.8	38 ± 5*	54.9
Anti-Lyt 1.1 + complement	5 ± 1	38.2	19 ± 3	48.3
Anti-Lyt 2.1 + complement	40 ± 5*	4.1	36 ± 4*	8.5
Nonimmune splenocytes	4 ± 1	4.6	4 ± 1	3.9

[a] Splenocytes were taken from mice given HSV i.p. 6 weeks previously. The cells were stimulated in vitro for 5 days with UV-inactivated HSV, and dead cells were removed before antiserum and complement treatment; for each treatment, 5×10^6 cells were transferred.

[b] Average increase of the right footpad (given virus) over the left footpad (given HBSS). Groups of six mice per group were used. Values with an asterisk were significantly greater ($P < 0.01$) than those for the feet which received HBSS.

[c] Percent specific lysis of syngeneic HSV-infected L cells during a ^{51}Cr release assay. Lysis of control uninfected cells and allogeneic virus-infected cells was in all cases less than 10%.

TABLE 3. Comparison of viral clearance activity of splenocytes and T-cell subsets from mice 7 days after s.c. infection and 6 weeks after i.p. infection

Cell treatment[a]	Avg PFU per pinna[b]	
	7-day immune (s.c.)	6-wk immune (i.p.)
None	47,000	47,000
Complement	30	65
Anti-Lyt 1.1 + complement	11,000	730
Anti-Lyt 2.1 + complement	33	14,500

[a] For each treatment, 3×10^7 cells were used.

[b] Groups of three mice were given 60 mg of cyclophosphamide per kg 24 and 48 h before receiving 10^6 PFU of HSV in the pinna; 2 h later the cell transfers were given intravenously. Ears were removed 72 h later for measurement of infectivity. Two repeat experiments provided similar data.

Splenocytes and T-lymphocyte subsets from animals infected i.p. 6 weeks previously were tested for their ability to clear virus before in vitro stimulation with virus. Although the population showed virtually no CTL activity and was incapable of transferring DTH (data not shown), viral clearance was effected (Table 3). Furthermore, the cell population active in viral clearance in this instance was the Lyt 2^+ subset. Presumably the Lyt 2^+ HSV-specific CTL precursors differentiate rapidly enough upon adoptive transfer in vivo to effect viral clearance.

DISCUSSION

Our work confirms the previous reports that only the Lyt 1^+2^- T-lymphocyte subset mediates the DTH response against HSV in mice (8, 10). This contrasts with influenza in which both Lyt 1^+ and Lyt 2^+ cells can transfer DTH after live virus infection (7) and with lymphocytic choriomeningitis virus in which the cell type mediating DTH is restricted by the class I major histocompatibility complex and is presumably a Lyt 2^+ subset (17). Subset analysis was performed both on lymphoid cells taken from animals 1 week after in vivo infection as was done by previous investigators and on cell populations that had received secondary in vitro restimulation with virus. This latter memory cell population expressed high levels of *H-2* restricted cytotoxicity mediated by Lyt 2^+ lymphocytes and in addition was more effective at transferring DTH than were cells taken 7 days after in vivo infection. In the memory population, however, only the Lyt 1^+ cell population transferred DTH.

TABLE 4. Clearance of virus from infected pinnae by secondary HSV immune splenocytes and by T-cell subsets

Cells transferred	PFU per pinna for expt no.:[a]				
	1	2	3	4	5
None	25,000	5,300	500,000	4,500	8,000
3×10^7 secondary splenocytes	20	100	400	<10	10
3×10^7 secondary splenocytes treated with anti-Lyt 1.1 + complement	ND[b]	5,600	5,600	12	10
3×10^7 secondary splenocytes treated with anti-Lyt 2.1 + complement	ND	300	300	<10	20
3×10^7 secondary splenocytes treated with anti-Thy 1.2 + complement	20,000	ND	ND	ND	ND

[a] For protocol see Table 3, footnote *b*.

[b] ND, Not done.

TABLE 5. Activity of T-cell subsets from secondary HSV immune splenocytes for clearing virus from pinnae after adoptive cell transfer

No. of cells transferred	Avg PFU per pinna	
	Anti-Lyt 1.1 + complement	Anti-Lyt 2.1 + complement
None	8,000	8,000
1×10^7	10	20
5×10^6	40	40
1×10^6	10,000	950
5×10^5	8,000	550

The principal reason for performing our studies was to analyze the activity of different T-lymphocyte subsets at clearing virus from the local site of infection. At least two groups had, by adoptive transfer techniques, shown that clearance of virus was an exclusive property of the DTH-mediating Lyt 1^+2^- T lymphocytes (8, 10), implying that the Lyt 2^+ subset had no role to play in immunity. In contrast, Larsen et al. had shown previously with memory populations of HSV immune lymphocytes that protection against lethal challenge by HSV was carried out by Lyt 2^+ cells (5). Furthermore, Sethi et al. demonstrated that cloned populations of HSV-specific CTL mediated protection from a lethal challenge by HSV (15). Thus, Lyt 2^+ T cells are involved in immunity, but they may function solely to prevent invasion of critical target organs such as the central nervous system. In the present report we demonstrate that protection against such invasion is not the only function of Lyt 2^+ cells since these cells could also clear virus from the local site of infection. Why previous workers failed to show such an effect was presumably because of the nature of the cell transfers being investigated. Thus, the lymph node cells used by Nash and colleagues expressed no Lyt 2^+ CTL activity since the necessary period of prior in vitro culture was not performed before adoptive transfer (12). In contrast, the cells we used for adoptive transfer expressed potent CTL activity. However, at least with some lymphoid populations it may not be necessary to transfer active Lyt 2^+ CTL to effect viral clearance. For instance, splenocytes from animals infected with virus 6 weeks previously and unable to adoptively transfer DTH or express appreciable CTL activity could still mediate viral clearance. Moreover, the cell population effective at clearance was a Lyt 2^+ subset, not the Lyt 1^+ subset responsible for clearance in lymphoid populations from animals recently infected with virus. This could mean that the Lyt 2^+ CTL precursors differentiate rapidly enough upon viral stimulation in vivo to become effective at clearance, whereas the precursors of Lyt 1^+ cells were unable to do so. Experiments are in progress to assess these possibilities.

The final aspect of our study was the observation that infection by the i.p. route, as with infection by the intravenous route noted by others (11, 14), led to a suboptimum stimulation of the DTH response. In fact, the DTH response in animals infected i.p. was always lower than in those infected s.c. and was often undetectable. Moreover, splenocytes from animals given virus i.p. usually failed to adoptively transfer DTH. However, the i.p. route of infection does establish Lyt 1^+ DTH-mediating memory, since if cells from animals infected 6 weeks previously were stimulated in vitro

with virus for 5 days, potent DTH-mediating activity could be demonstrated. Whether this means that under conditions of in vitro culture the HSV-specific Lyt 1^+ precursors are able to differentiate because suppressor cell effects are being abrogated in some way is currently under investigation.

ACKNOWLEDGMENTS

The superb technical assistance of Linda Miller was appreciated.

This work was supported by grant AI 14981 from the National Institute of Allergy and Infectious Diseases.

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