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Immunity to Herpes Simplex Virus Type 2: Cell-Mediated Immunity in Latently Infected Guinea Pigs

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Cell-mediated (CMI) and humoral immunity to herpes simplex virus type 2 (HSV-2) were evaluated in infected strain 13/N guinea pigs with (45%) and without a history of recurrent herpetic disease. Virus was isolated by cocultivation from active herpetic lesions (9 of 10) as well as from the footpads (17 of 38), sacral ganglia (7 of 21), and sciatic nerves (1 of 21) of asymptomatic animals. Viral isolates grew in cells of human origin and were neutralized by hyperimmune anti-HSV-2 sera. Humoral immunity measured by the presence of virus-neutralizing antibody was similar in both experimental groups. The involvement of CMI in recurrent disease was assessed by comparing lymphocyte transformation (LT) and leukocyte migration inhibition factor (LIF) responses in animals with a history of recurrent disease studied while asymptomatic (quiescent) and in animals without clinical evidence of recurrent disease (seropositive controls). Spleen cells from quiescent animals evidenced significant impairment of both LIF and LT responses as reflected in the requirement of higher antigen concentrations (up to 58-fold) and longer in vitro culture periods (up to 2.5 days) to mount responses comparable in magnitude to those observed in the seropositive control groups. Peripheral blood lymphocyte cultures obtained from quiescent animals showed similar impairment of LIF responses but displayed intact LT response. The data suggest that recurrent disease is associated with an impairment in the generation of anamnestic effector functions as reflected by altered kinetics and dose response patterns in in vitro secondary responses.

The hallmark of herpetic disease in humans is the ability of the virus to cause recurrent disease in a proportion of infected individuals. Although it was early recognized that recurrent disease occurs as a consequence of the reactivation of latent virus (11), the mechanisms of virus persistence and reactivation have yet to be determined. The observation that individuals with impaired cell-mediated immunity (CMI) often suffer severe herpetic infections of apparently endogenous origin (1, 22) has led to the hypothesis that recurrent herpes is associated with a defect in CMI. However, available evidence is equivocal. Thus, individuals with recurrent disease display intact virus-specific lymphoproliferation (23, 31, 32, 36, 40), natural killer cell-mediated cytotoxicity (13, 28), antibody-dependent cell cytotoxicity (13), and B-cell-mediated interferon release (27). However, recurrent disease has been associated with depressed T-cell-mediated effector responses such as leukocyte migration inhibition factor (LIF) (25, 36) or macrophage migration inhibition factor (MIF) (14, 37, 40).

The present studies were made possible by the development of the guinea pig model of recurrent herpetic disease (34), a model particularly well suited for the study of CMI since immune regulation in this animal closely resembles that in humans (7). We have previously characterized the evolution, in guinea pigs, of the virus-specific immune response after primary HSV-2 infection in terms of both the kinetics and the dose requirements of in vitro lymphoproliferation and LIF activity (8). Having applied the same analyses to long-term-infected animals with and without a history of recurrent disease, the studies described in this report demonstrate that recurrent disease is, indeed, associated with an impaired CMI response.

MATERIALS AND METHODS

Cells. Human epidermoid carcinoma no. 2 (HEp-2) cells (Microbiological Associates, Bethesda, Md.) were propagated in medium 199 with 10% calf serum. Primary guinea pig embryo (GPE) cells, prepared from 13/N fetuses (15 to 25 days of gestation), and primary guinea pig kidney (GPK) cells (Flow Laboratories, Inc., Rockville, Md.) were grown in Eagle minimal essential medium with 10% fetal calf serum (MEM-10% FCS).

Viruses. The isolation and properties of the G

strain of herpes simplex virus type 2 [HSV-2 (G)] were previously described (10). It was plaque purified and passaged in GPE cells before use in these studies. HSV (MP), a type 1 virus that antigenically behaves as an intermediate between HSV-2 and HSV-1 (10), gives rise in HEp-2 cells to characteristic polykaryocytes readily differentiated from the cell clumps caused by HSV-2 (G) (2, 3,10).

Animal inoculation. Inbred strain 13/N guinea pigs originally obtained from C. K. Hsu (Frederick Cancer Research Center, Frederick, Md.) were bred in closed colony in our laboratory. Animals of both sexes ranging in weight from 150 to 950 g (mean = 500 g ± 25) were injected subcutaneously in the left hind footpad with 10^4 plaque-forming units (PFU) of HSV-2 (G) (60 μl). A single virus preparation was used throughout these experiments (8).

Virus neutralization. Antibody assays were performed by plaque reduction multiplicity analysis as described (2), using artificial mixtures of HSV-2 (G) and HSV (MP). The relative extent of neutralization was expressed as K value calculated from the formula $\log V/V_0 = -0.43\ K_c$, where V/V_0 is the proportion of virus surviving neutralization and c is the serum concentration expressed as the amount of undiluted serum (microliters) present in 1.0 ml of reaction mixture. In this assay, the ratio of the K values [$r = K_{HSV\text{-}2}/K_{HSV\ (MP)}$] reflects antibody specificity; a ratio of ≥1 indicating a type 2 virus response (2). Since assays were performed simultaneously with the same virus preparation, K values can be accepted as indicative of the relative levels of virus-specific antibody in the sera (2). The sensitivity and specificity of this assay for virus typing have previously been described (3, 10, 39).

Virus isolation. Tissue specimens used in virus isolation included: (i) 4-mm section of the sciatic and tibial nerves taken from the flexture of the knee, (ii) 2- by 4-mm section of the tarsal footpad, and (iii) dorsal root ganglia (L_4-S_3). In the cocultivation assay, minced tissue specimens were plated on monolayers of GPK cells in MEM-2% FCS. Cultures were refluided weekly and maintained for at least 4 weeks. In the homogenate procedure, minced tissue specimens were made into an approximately 10% (wt/vol) suspension in MEM, mixed with an equal volume of sterile glass beads (0.5 mm; Heat Systems Co., Melville, N.Y.), and exposed to 3- to 30-s cycles of sonication (W185 Heat Ultrasonics sonic oscillator), each followed by one cycle of freezing and thawing. Supernatants obtained after centrifugation for 10 min at 2,000 × g were used to infect GPK cells. Since guinea pig herpesviruses do not grow in cells of human origin (19), all isolates were passaged at least once in HEp-2 cells.

Antigens. HSV-2 (G) passaged twice in 13/N GPE cells at 0.2 PFU/cell was used in antigen preparation as previously described (8). Virus was pelleted from the supernatant (extracellular) and the extract of the infected cells (intracellular) by centrifugation at 100,000 × g for 1 h. It was resuspended in phosphate-buffered saline (PBS) to final concentrations of 6.6×10^7 PFU (100 μg of protein/ml) and 3.2×10^7 PFU (240 μg of protein/ml) for extracellular and intracellular virus, respectively, and inactivated by exposure to ultraviolet (UV) irradiation at 17 cm from a Sylvania G15T8 source for 30 min.

Lymphocyte preparations. Splenic mononuclear cell preparations (SC) were obtained as described (8). Peripheral blood lymphocytes (PBL) were prepared from heparinized (20 U/ml; The Upjohn Co., Kalamazoo, Mich.) blood, obtained by cardiac puncture, mixed with equal volumes of calcium and magnesium-free PBS (Dulbecco PBS-A) and centrifuged at 800 × g for 30 min on a gradient of 5 parts of 32.8% (wt/vol) sodium metrizoate (Gallard-Schlesinger, Westbury, N.Y.), and 12 parts of 12% (wt/vol) Ficoll 400 (density = 1.09 g/ml). Mononuclear cells were collected from the interface. Platelets were removed by washing three times with medium RPMI 1640 at low speed for 2 min.

In vitro culture of lymphoid cells. Cells (2.5×10^6 cells/ml) were cultured at 37°C in freshly made RPMI 1640 medium with 10% decomplemented normal 13/N guinea pig serum, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, 5×10^{-5} M 2-β-mercaptoethanol, 10 U of mycostatin per ml, and 50 μg of gentamicin per ml as previously described (8). Cultures of SC (1 ml) and PBL (0.5 ml) were set in quintuplicate in snap-cap tubes (12 by 75 mm; Falcon 2054) in the presence of antigen or PBS control (0.1 and 0.05 ml for SC and PBL cultures, respectively). At harvest, SC were resuspended by Vortex mixing, and cell samples (100 μl) were transferred in triplicate to microtiter wells (Falcon 2040) for lymphocyte transformation (LT) assay. SC remaining in the culture tubes were centrifuged at 500 × g for 10 min, and the supernatants were collected for LIF assay. PBL were first centrifuged at 500 × g for 10 min, and the supernatants were removed for use in LIF assay. Portions (100 μl) of the cells resuspended in 0.5 ml of fresh RPMI 1640 with 10% 13/N guinea pig serum were then transferred in triplicate to microtiter wells for LT assay.

LT assay. The assay was performed as described (8). Cells were pulsed for 4 h at 37°C with 25 μl of tritiated thymidine ([^{3}H]TdR; 40 μCi/ml) per well and harvested onto glass fiber filters (Reeve Angel grade 934 AH). Results were expressed as net cpm = (mean $cpm_{experimental}$) − (mean $cpm_{PBS\ control}$). Based on previously described specificity assays using mock antigen prepared in identical fashion from uninfected cells, a response was considered positive if it registered ≥200 net cpm. Responses to mock antigen in a series of control experiments were 38.3 net cpm where upper and lower 99% confidence intervals were 81.0 and 0.0 net cpm, respectively (8).

LIF assay. A previously described (8) modification of the indirect microdroplet procedure (16) was used. Supernatants of SC and PBL cultures were supplemented with 10% decomplemented FCS and assayed for LIF activity on polymorphonuclear leukocytes (PMNL) prepared from uninfected animals as described (8). Microtiter wells containing droplets of cells (4×10^5 PMNL) in medium 199–10% syngeneic serum–0.2% agarose were overlayed with supernatants of SC or PBL cultures and incubated for 16 h at 37°C. Cells were fixed, agarose droplets were aspirated, and the area of PMNL migration was measured blindly by planimetry. The net area of migration was determined for each well by subtracting the area occupied by the agarose droplet from the total area of migration. Migration inhibition index (MI) was calculated from the

formula:

$$MI = \frac{\text{mean net migration}_{\text{experimental}}}{\text{mean net migration}_{\text{control}}} \times 100$$

All supernatants were assayed in triplicate. Based on data generated with mock antigen control, an MI of 80 was considered positive. The mean migration in response to mock antigen was 97.8 with upper and lower 99% confidence intervals of 100.4 and 95.2, respectively (8).

RESULTS

13/N guinea pig model of HSV-2 recurrent disease. Guinea pigs (79 females and 69 males) were infected with HSV-2 (G) in seven cohorts comprised of 20 to 28 animals. They were monitored daily for 2 weeks postinfection (p.i.) and one to two times per week thereafter. This protocol was selected on the basis of data generated by daily examination of the first 20 animals for a period of 3 months, indicating that the majority of recurrences last at least 1 week. The infected footpad was scored for erythema and swelling on a scale of none, mild, moderate, and marked. Mild or moderate erythema was occasionally observed in uninfected footpads from older animals but never in conjunction with swelling. Erythema alone, regardless of degree, was not scored.

Clinical symptoms consisting of at least moderate erythema with swelling occurred within the first 24 to 48 h after primary infection in 63% of the animals. Symptomatology correlated well with the weight (hence the age) of the animals at the time of infection. Thus the mean weight (±1 standard error) of 72 animals with relatively severe primary disease was 550 ± 25 g as compared with 400 ± 25 g in 53 animals that displayed relatively mild symptoms (Student t test, $P < 0.001$). Primary infection occurred without symptoms in five of five weanling guinea pigs (4 to 6 weeks age; mean weight = 150 g), and severe symptoms were observed in all of 18 guinea pigs that weighed >700 g at the time of infection. The incidence of clinically apparent primary infection was independent of sex ($\chi^2 = 0.53$, $P > 0.25$). The mean duration of symptoms was 8.4 ± 1.1 days. However, this statistic includes four animals with symptoms that persisted for 38 to 50 days, possibly representing superimposed recurrent disease.

Recurrent disease was defined as the reappearance of symptoms (at least moderate erythema with swelling) after one or more intervals of negative observation. In the case of those animals that did not evidence primary disease, the first observed lesion was classified as recurrent if the symptoms first occurred at least 3 weeks after animal inoculation. Consistent with Scriba's findings (34), recurrent disease was usually accompanied by hemorrhagic macules. Marked swelling or hemorrhagic lesions were never observed in uninfected footpads. Most (33 of 148) of the animals with recurrent disease developed their first episode during the 1st month p.i. The number of animals experiencing their first recurrent lesions during the 2nd, 3rd, and 4th months p.i. was 17 of 131 (13%), 6 of 105 (5.7%), and 1 of 83 (1.2%), respectively. The proportion of animals with at least one episode of recurrent disease did not increase beyond 8 months p.i. (Table 1). Among animals with recurrent disease, the mean number of episodes was 4.0 ± 0.3 recurrences per year, and their mean duration was 13 days. The incidence of recurrent disease did not correlate with: (i) weight at infection (mean weight recurrent = 525 ± 25 g; mean weight nonrecurrent = 550 ± 25 g; $P > 0.4$), (ii) clinical course of primary infection ($\chi^2 = 0.12$; $P > 0.5$), or (iii) sex ($\chi^2 = 0.53$; $P > 0.25$).

Thirty-six sera from animals at 2 and 15 months p.i. were assayed for virus-neutralizing antibody. Twenty-one were from animals that had experienced clinically apparent primary infection, 13 were from animals with a history of recurrent disease bled during quiescence, and 23 were from animals that had never experienced recurrent disease. All sera were positive ($r \geq 1$) for neutralizing antibody to HSV-2 (Table 2). Significant differences in antibody levels were not observed between animals that did (mean $K_{HSV-2} = 0.32$) or did not (mean $K_{HSV-2} = 0.36$) experience symptomatic primary infection ($P = 0.1$). However, K_{HSV-2} values of sera from quiescent animals were slightly but significantly ($P < 0.05$) higher than those of sera from animals without a history of recurrent disease, henceforth designated seropositive controls.

The frequency of virus isolation from asymptomatic animals was higher by the cocultivation than by the isolation procedure from cell-free homogenates (Table 3). Consistent with the for-

TABLE 1. *History of recurrent disease in HSV-2 (G)-infected animals*

Months p.i.	No. of animals	No. (%) with first episode of recurrent disease	% with history of recurrent disease[a]
0–1	148	33 (22.3)	22.3
1–2	131	17 (13.0)	35.3
2–3	105	6 (5.7)	41.0
3–4	83	1 (1.2)	42.2
4–8	65	2 (3.1)	45.3
8–12	25	0 (0.0)	45.3
>12	19	0 (0.0)	45.3

[a] Percentage of total number of studied animals evidencing at least one episode of recurrent disease between the time of infection and the stated time.

mer's increased sensitivity (34, 35, 38), virus was isolated by cocultivation of sacral ganglia and foot tissue (site of infection) from approximately one-half of the asymptomatic animals (Table 3). The isolates were not endogenous guinea pig herpesviruses since: (i) they grew in cells (HEp-2) that are nonpermissive for the guinea pig herpesviruses (19) and (ii) all 12 isolates typed in this series were preferentially neutralized by hyperimmune rabbit anti-HSV-2 (G) serum.

LT response in SC cultures. SC from 6 quiescent and 13 seropositive controls were exposed to increasing concentrations (0.003, 0.01, 0.05, 0.19, 0.75, 3.0, and 12.0 μg of protein/ml) of intracellular UV-treated HSV-2 (G) and assayed for [^{3}H]TdR incorporation on days 1, 2, 3, 4, and 6 in culture. Dose-response curves generated at 2 and 6 days in culture (plotted on a logarithmic scale for ease of comparison) and in vitro kinetics in response to high antigen dose (12 μg of protein/ml) are shown in Fig. 1. Both groups of animals mounted significant LT responses. However, whereas SC from seropositive controls exposed to the highest antigen dose (12.0 μg/ml) displayed a minimal response (300 ± 100 cpm) after 1 day in culture, SC from quiescent animals were unresponsive at this time. Differences in dose responses were most pronounced at 2 days in culture (Fig. 1A). At this time it can be graphically estimated that a 58-fold-higher antigen concentration (2.3 versus 0.04 μg/ml) is required to elicit a minimal positive response (200 net cpm) in SC cultures from quiescent animals as compared with seropositive controls. Although differences were somewhat less pronounced at 6 days in culture (Fig. 1B), SC from quiescent animals still required >16-fold-higher antigen concentrations in order to mount responses equivalent to those obtained in SC cultures from seropositive controls. Significantly, the two groups also differed in the magnitude of the response. At 12.0 μg of antigen protein per ml, the day 6 response was fourfold greater (80,000 versus 18,000 net cpm) in the seropositive controls.

In both experimental groups, LT responses increased exponentially as a function of time in culture and thus were linear when plotted on a log-arithmetic scale (Fig. 1C). The lines of best fit as determined by the method of least squares are shown. At the highest antigen dose tested (12.0 μg/ml), a positive response was first observed in the seropositive control group as early as 1 day in culture; however, SC from quiescent animals did not respond at that time. The time required to generate a minimal positive response (200 net cpm) in the presence of 12.0 μg of protein of UV-treated HSV-2 (G) per ml can be graphically estimated as 0.4 days for SC from seropositive controls as compared with 2.1 days for SC obtained from quiescent animals (Fig. 1C). The difference in the kinetics of the response was more pronounced at the low antigen concentrations. Thus, at 0.05 μg of protein per ml, a positive response was first seen at 1.5 days in cultures of SC from seropositive controls but not until 3.9 days in cultures of SC from quiescent animals (data not shown). It should be pointed out that despite these differences, the slopes of the responses were indistinguishable at all antigen doses. Accordingly, the doubling time, defined as the time of in vitro exposure to

TABLE 2. *Virus-neutralizing antibody in HSV-2 (G)-infected guinea pigs*

History of recurrent disease[a]	No. of sera assayed	Mean[b] K_{HSV-2} (μl^{-1})	Mean[c] K_{HSV-1} (μl^{-1})	Mean[d] r
Negative	23	0.31 ± 0.02	0.22 ± 0.01	1.42 ± 0.05
Positive (Quiescent)	13	0.39 ± 0.04	0.31 ± 0.03	1.27 ± 0.09

[a] Animals without a history of recurrent disease (negative) and those with a history of recurrent disease bled while asymptomatic (quiescent) were studied at 2 to 15 months p.i.

[b] Levels of antibody to HSV-2 (K_{HSV-2}) ± 1 standard error are shown. Responses are significantly higher (Student t test, $P < 0.05$) in the quiescent animals than in those negative for recurrent disease.

[c] Levels of cross-reacting antibody to HSV-1 (K_{HSV-1}) are significantly higher in the quiescent animals than in those negative for recurrent disease ($P < 0.01$ by Student t test).

[d] $r = K_{HSV-2}/K_{HSV-1}$ is consistently >1.0, indicative of an HSV-2 response (2), and is not significantly different in the two groups of animals.

TABLE 3. *Virus isolation from HSV-2 (G)-infected animals*

Tissue origin	Homogenate assay[a]			Cocultivation procedure[a]		
	Recurrence negative	Recurrence positive		Recurrence negative	Recurrence positive	
		Quiescent	Active		Quiescent	Active
Footpad	1/7 (14)	1/8 (13)	3/3 (100)[b]	8/21 (38)	9/17 (53)	9/10 (90)
Sacral ganglia	0/7 (0)	0/8 (0)	0/1 (0)	2/10 (20)	5/11 (45)[c]	0/5 (0)[c]
Sciatic nerve	0/7 (0)	0/8 (0)	0/1 (0)	0/10 (0)	1/11 (9)	0/5 (0)

[a] Number with positive isolates over total number tested. Parentheses represent percentage.

[b] Two specimens were obtained by biopsy.

[c] Difference not statistically significant ($\chi^2 = 2.05$; $P > 0.1$).

antigen required in order to double the magnitude of [^{3}H]TdR incorporation and calculated as previously described (8), was the same (0.6 days) in both groups.

LIF responses in SC cultures. Supernatants from cultures of SC obtained from seropositive controls displayed a positive LIF response of low magnitude (MI ≤ 80) as early as 1 day in culture at high (3.0 to 12.0 μg of protein/ml) antigen concentrations. Marginal (MI = 81 ± 8) LIF response was observed in supernatants of day 1 cultures of SC from quiescent animals but only at 12.0 μg of antigen protein per ml (Fig. 2A). The greatest difference between the two groups was observed at 3 days in culture. At this time, a minimal positive response (MI = 80) was attained in the seropositive control group at 0.4 μg of protein per ml, whereas the quiescent group was unresponsive throughout (Fig. 2B). However, given sufficient time in culture (6 days), SC from quiescent animals mounted a significant LIF response at antigen concentrations as low as 0.16 μg of protein per ml. At this time, the responses in the two groups overlapped at the highest (0.75 to 12.0 μg of protein/ml) antigen concentrations (Fig. 2C).

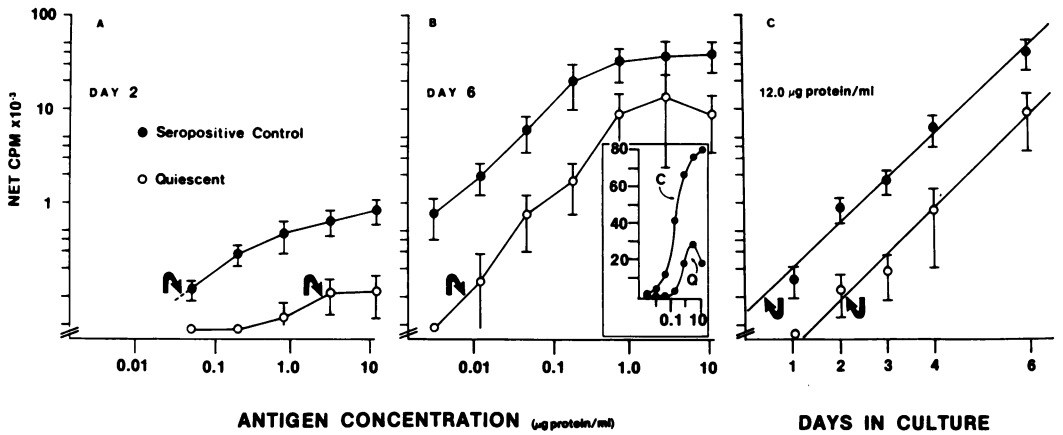

FIG. 1. *Dose response and in vitro kinetics of [^{3}H]TdR incorporation in cultures of SC obtained from seropositive control (●) and quiescent (○) guinea pigs. (A, B) Effect of antigen [UV-treated HSV-2 (G)] dose in 2- and 6-day cultures. (C) In vitro kinetics in cultures exposed to high (12 μg of protein/ml) UV-treated HSV-2 (G) antigen concentration. Antigen concentration and net cpm (×10^{-3}) (means ± 1 standard error) are plotted on a logarithmic scale. Arrows indicate dose required to elicit a minimal positive response (200 net cpm). Insert (panel B) represents the response at 6 days in culture plotted as previously described (8) on an arithmetic-log scale. C, seropositive controls; Q, quiescent.*

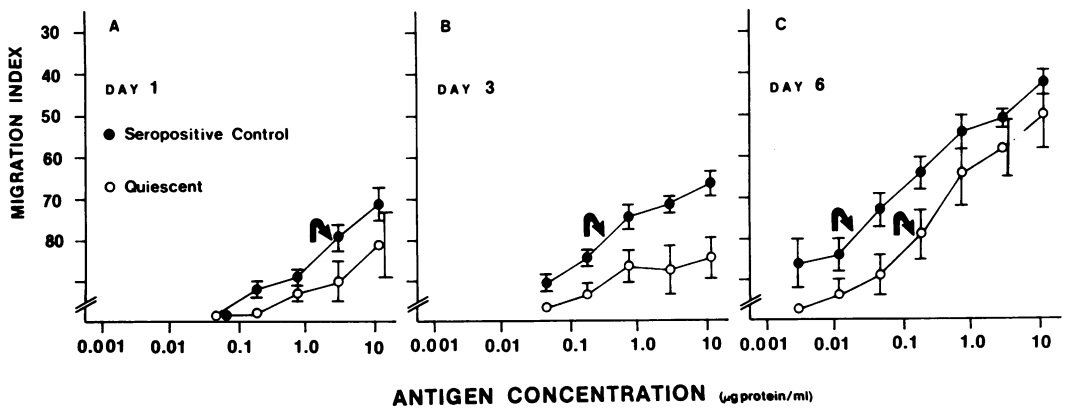

FIG. 2. *Effect of antigen dose on LIF production in SC cultures. SC obtained from seropositive control (●) and quiescent (○) animals were cultured in presence of increasing doses of UV-treated HSV-2 (G) antigen. Supernatants were harvested at 1 (A), 3 (B), and 6 (C) days in culture and assayed for LIF activity. Antigen concentration is plotted on a logarithmic scale. Analysis also includes animals not assayed in LT for a total of 8 quiescent and 18 seropositive controls.*

As previously reported (8), LIF production in cultures of SC from seropositive controls exposed to high (12.0 μg/ml) doses of UV-treated HSV-2 (G) antigen was biphasic and consisted of an early component released during the first 24 h in culture and a late component elaborated from 3 to 6 days in culture (Fig. 3A). Although the mean early response was marginal (MI = 81) in cultures of SC from quiescent animals (Fig. 3A), the observation that a dose-response relationship obtains at 1 day in culture (Fig. 2A) suggests that low LIF levels are indeed produced at that time. At intermediate (0.75 μg of protein/ml; Fig. 3B) and low (0.05 μg of protein/ml; Fig. 3C) antigen concentrations, only the late component of the LIF response was produced with a lag of at least 1 day in the quiescent as compared with the seropositive control group. The LT and LIF responses in the SC cultures from the two experimental groups are summarized in Table 4.

LT and LIF responses in PBL cultures. PBL from five quiescent and eight seropositive control animals were cultured in presence of extracellular UV-treated HSV-2 (G) antigen at concentrations (0.001, 0.005, 0.02, 0.31, and 5.0 μg of protein/ml) based on the observation that responses obtained with 5 μg of this antigen per ml were identical to those obtained with 12.0 μg of intracellular UV-treated HSV-2 (G) antigen per ml. Lymphoproliferative responses in cultures of PBL from quiescent and seropositive

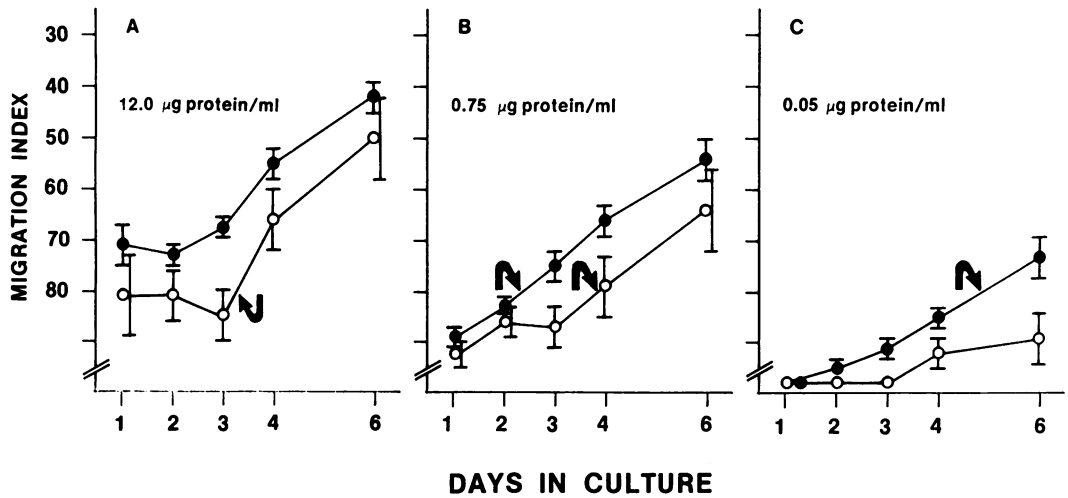

FIG. 3. *In vitro kinetics of LIF response in SC cultures. SC obtained from seropositive control (●) and quiescent (○) animals were cultured in the presence of 12.0 (A), 0.75 (B), and 0.05 (C) μg of protein of UV-HSV-2 (G) antigen per ml. Culture supernatants were assayed for LIF activity. Arrows indicate dose required to elicit a minimal positive response (MI = 80).*

TABLE 4. *Summary of LT and LIF responses in SC culture*

Group	Onset (days) of[a]:				Minimal dose (μg/ml) for[b]:				
	LT, antigen dose of 0.05 μg/ml	Early LIF, antigen dose of 12.0 μg/ml	Late LIF, antigen dose of:		LT		Early LIF, 1 day	Late LIF	
			0.75 μg/ml	0.06 μg/ml	2 days	3 days		3 days	6 days
Seropositive controls	1.5	<1	1.4	4.9	0.04	≪0.05	2.6	0.4	0.02
Quiescent	3.9	<1	3.9	No response	2.3	0.13	12.0	>12.0	0.16
Difference or ratio (fold)	2.4	Nil	2.5		58	≫2.7[c]	4.6	>30	8

[a] Period of in vitro culture required to elicit a minimal positive LT (net cpm = 200) or LIF (MI = 80) response at the stated antigen concentration. All values determined graphically from data shown in Fig. 3 and in part in Fig. 1.

[b] Antigen dose required to elicit a minimal positive response at stated time in culture. Values were determined from data shown in Fig. 2 and in part in Fig. 1.

[c] Based on graphic extrapolation of the data for seropositive controls, the ratio is approximately 63-fold.

control animals were indistinguishable both in terms of dose-response and in vitro kinetic (Fig. 4) analyses. Unlike the SC observations, the slope of the LT response (Fig. 4) appeared to decrease as a function of time in culture.

PBL from seropositive control but not from quiescent animals were positive for early LIF when exposed to high (5.0 μg of protein/ml) antigen concentrations (Fig. 5A). It can be graphically estimated (Fig. 5B) that at 3 days in culture (late LIF), the dose of antigen required to elicit a minimal positive response (MI = 80) in PBL cultures from seropositive control animals is 0.54 μg of protein per ml. At this time, PBL from quiescent animals were unresponsive. However, at 6 days in culture (Fig. 5C), PBL from quiescent animals exposed to 5.0 μg of protein of UV-treated HSV-2 (G) antigen per ml evidenced a positive LIF response. As in SC cultures, LIF response in cultures of PBL from quiescent animals lagged behind those observed in cultures of PBL from seropositive controls by at least 1 day (Fig. 6). The LT and LIF responses in PBL cultures from the two experimental groups are summarized in Table 5.

DISCUSSION

The salient feature of these studies is the observation that recurrent disease is associated with an impaired virus-specific CMI response. The results merit discussion from the standpoint of the fidelity of the animal model and the

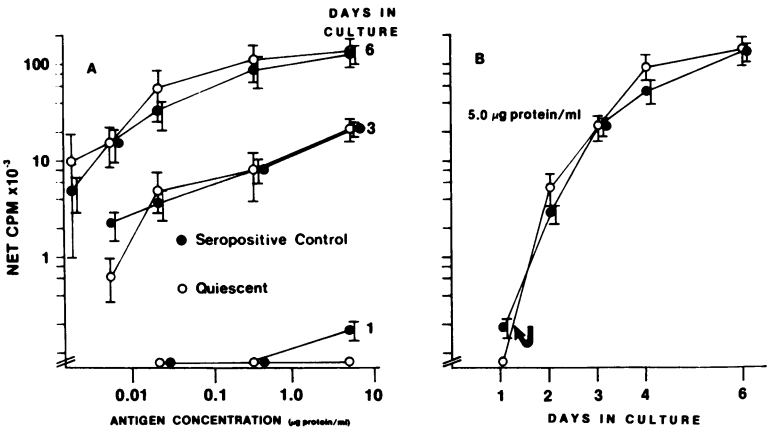

FIG. 4. *Dose response and in vitro kinetics of [^{3}H]TdR incorporation in cultures of PBL obtained from seropositive control (●) and quiescent (○) guinea pigs. (A) Effect of antigen dose [extracellular UV-treated HSV-2 (G)] on [^{3}H]TdR incorporation in 1-, 3-, and 6-day cultures. (B) In vitro kinetics of [^{3}H]TdR incorporation in PBL cultured in presence of 5.0 μg of protein of extracellular UV-treated HSV-2 (G) antigen. Arrow indicates time in culture required to elicit a minimal positive response (200 net cpm).*

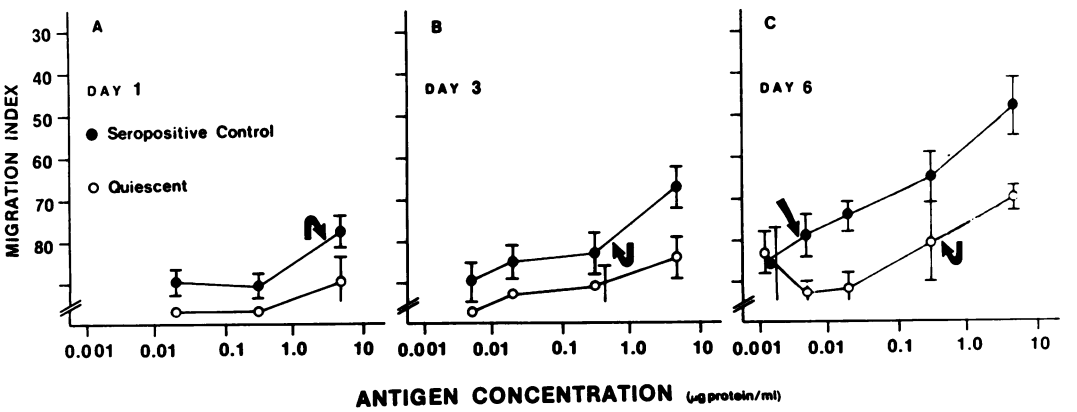

FIG. 5. *Effect of antigen dose on LIF production in PBL cultures. PBL obtained from seropositive control (●) and quiescent (○) guinea pigs were cultured in presence of increasing doses of extracellular UV-treated HSV-2 (G) antigen. Supernatants were harvested at 1, 3, and 6 days in culture and assayed for LIF activity. Arrows indicate dose required to elicit a minimal positive response (MI = 80).*

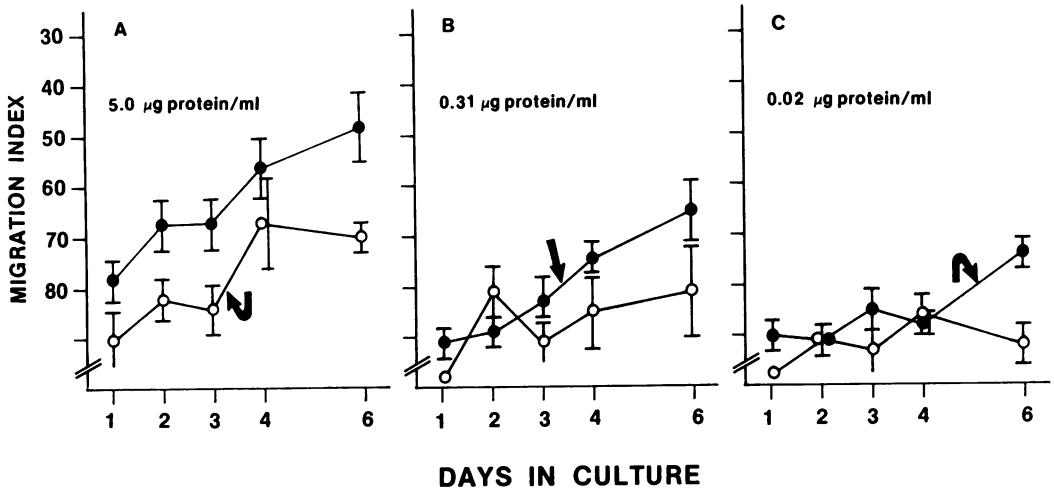

FIG. 6. *In vitro kinetics of LIF response in PBL cultures. PBL obtained from seropositive control (●) and quiescent (○) guinea pigs were cultured in the presence of 5.0 (A), 0.31 (B), and 0.02 (C) μg of protein of extracellular UV-treated HSV-2 (G) antigen per ml. Supernatants were assayed for LIF activity at 1, 2, 3, 4, and 6 days in culture. Arrows indicate time in culture required to elicit a minimal positive response (MI = 80).*

TABLE 5. *Summary of LT and LIF responses in PBL cultures*

Group	Onset (days) of[a]:				Minimum dose (μg/ml) for[b]:			
	LT, antigen dose of 0.02 μg/ml	Early LIF, antigen dose of 5.0 μg/ml	Late LIF, antigen dose of:		LT, 3 days	Early LIF, 1 day	Late LIF	
			0.31 μg/ml	0.02 μg/ml			3 days	6 days
Seropositive controls	1.4	<1	3.4	5.1	<0.005	3.3	0.54	0.004
Quiescent	1.4	NR[c]	>5.0	NR	<0.005	NR	>5.0	0.41
Difference or ratio (fold)	Nil		>1.6		Nil		>9.3	102.5

[a] Period of in vitro culture required to elicit a minimal positive LT (net cpm = 200) or LIF (MI = 80) response at the stated antigen concentration. All values determined graphically from data shown in Fig. 6 and in part in Fig. 4.

[b] Antigen dose required to elicit a minimal positive response at stated time in culture. Values were determined from data shown in Fig. 5 and in part in Fig. 4.

[c] NR, No response.

possible role of CMI responses in recurrent disease.

Animal model. Consistent with Scriba's findings in the Hartley strain guinea pigs (34, 35), 13/N animals become latently infected with HSV-2 after footpad inoculaton. However, despite the fact that inbred animals were used and care was taken to ensure uniform infection, recurrent disease was observed in only 45% of infected animals. This proportion was highly consistent between cohorts of animals. The discrepancy between this observation and Scriba's report (34) that as many as 80 to 90% experience recrudescent lesions probably reflects the different virus strains used in the two studies. Thus, (i) only 50% of Hartley strain guinea pigs infected with the G strain of HSV-2 experience recurrent disease (Donnenberg and Aurelian, unpublished data) and (ii) Hartley as well as 13/N animals infected with HSV-1 strain MacIntyre (35) or F (Donnenberg and Aurelian, unpublished data) develop latent infection as evidenced by seroconversion, but do not experience clinically apparent recurrent disease. Three observations support the conclusion that those 13/N animals without recurrent disease have nevertheless been infected: (i) the majority of animals studied in these series (63%) developed clinically evident primary herpetic disease, (ii) all of the 23 randomly selected animals without a history of recurrent disease (including nine that had an asymptomatic primary infection) were HSV se-

ropositive as late as 15 months p.i. (Table 2), and (iii) infectious virus was successfully isolated from these animals in absence of clinically apparent lesions (Table 3). These results are consistent with our previous observations (8) indicating that at 150 days p.i., animals without recurrent disease are positive for early LIF, a response that depends on continuous antigen expression (5, 18).

Clinically overt primary cutaneous disease is highly correlated with the weight (hence the age) of the animal. Thus, whereas younger animals (<500 g) experienced a low (41%) incidence of symptomatic disease, clinically overt primary lesions were observed in 79% of the older (≥500 g) animals. This finding agrees with the observation that primary cutaneous HSV disease, though severe in adults, tends to be mild or entirely without symptoms in young children (6). The mechanism involved in this phenomenon is unknown. However the observation stands in contradistinction to the finding that in HSV animal models entailing severe sequelae such as encephalitis (17, 20) or hepatitis (24), young or neonatal animals have decreased survival times, increased mortality, and more pronounced histopathological changes than adults. Also consistent with human disease, neither the weight at primary infection nor the clinical course of the primary disease is predictive of the development of recurrent lesions, manifested with equal frequency by animals with and without a clinically apparent primary infection (6, 9, 21, 30). Furthermore, all the infected animals studied in these series were HSV-2 seropositive. Significantly, antibody titers expressed as K values were significantly higher in those animals with a history of recurrent disease (Table 2), possibly due to their periodic exposure to relatively large doses of viral antigen during recrudescence.

The virus isolates obtained at intervals p.i. are not guinea pig herpesviruses, since they survive passage in HEp-2 cells (19) and are preferentially neutralized by hyperimmune rabbit anti-HSV-2 serum. The relatively high frequency of virus isolation from asymptomatic 13/N animals could be interpreted as indicative of virus persistence as a chronic low-grade infection. As such, our data are consistent with Scriba's results from HSV-2-infected Hartley strain guinea pigs (34, 35) and with those obtained from various human studies (6, 9, 21), but they are at variance with Steven's (38) results in the HSV-1 mouse model. In the latter, virus was not isolated from the site of infection of asymptomatic animals, and original studies supported the interpretation that it persists as a repressed genome (26, 38). More recently, however, Schwartz et al. (33) described the isolation of infectious HSV-1 from dorsal root ganglion homogenates obtained from latently infected mice, thus supporting the interpretation that both virus serotypes can persist in neural tissue as a chronic low-grade infection. Virus persistence at the site of infection (footpad) was not found in the mouse HSV-1 model (38), a phenomenon possibly associated with the absence in this model of spontaneously occurring recurrent lesions.

CMI response in recurrent disease. SC from seropositive controls display a minimal positive LT response (200 net cpm) in the presence of low (0.05 μg/ml) antigen doses within 1.5 days in culture as compared with 3.9 days for the quiescent group (Table 5). The observation that the apparent difference in the kinetics of the response observed for the two groups is attributable to a shift in the y intercept of the curves rather than in their slopes (Fig. 1C) is amenable to several interpretations: (i) SC cultures from quiescent animals have fewer HSV-specific immune cells capable of proliferating upon contact with antigen, (ii) immune cells are present in adequate number but antigen processing or presentation by accessory cells is impaired, or (iii) a suppressive mechanism actively prevents proliferation of available immune cells. Regardless of the antigen dose, the slopes, and hence the doubling times, of SC lymphoproliferative responses were indistinguishable between the two groups.

SC cultures from both experimental groups mount early LIF (Fig. 3), a response that may reflect the presence of effector cells predifferentiated in vivo (8). However, a lag similar to that observed in the LT assay is seen in the time required for SC from quiescent animals to express late LIF (Fig. 3), a response that appears to require in vitro proliferation or differentiation (8). Furthermore, at early culture intervals, the dose required to elicit a minimal positive LT response is 64-fold higher than that required to elicit an equivalent response in the seropositive control group and >30-fold-higher antigen concentrations are required to elicit late LIF response at 3 days in culture (Table 4).

Due to experimental differences, responses in PBL and SC are not strictly comparable. However, the observed differences cannot be readily attributable to the relative potency of the antigen preparation and the volumes of the respective cultures. Thus in PBL cultures, LT responses are identical in both experimental groups (Table 5), whereas LIF responses in the quiescent group evidence impairment in the ki-

netic and dose-response characteristics comparable to or greater than those observed in SC cultures. Animals were not studied at the time of active recurrent disease or immediate convalescence, and therefore information on the chronological alteration in LIF responsiveness is not available. Nevertheless, our data are conceptually consistent with the findings that, in humans, lymphokine but not lymphoproliferative responses are suppressed in cultures of PBL from patients with recurrent herpetic disease (25, 37, 40). Conceivably, impaired lymphoproliferation in SC but not in PBL cultures from quiescent animals may reflect the presence in the spleen, but not the PBL, of virus-specific suppressor cells. Indeed, the spleen has been identified as a preferential site of suppressor activity (15, 29). However, this interpretation predicts that LIF production in PBL cultures from quiescent animals should be equally unimpaired, a postulate not supported by the data summarized in Table 5. The simplest interpretation is that, compared with seropositive controls, quiescent animals have fewer cells in the peripheral circulation committed to LIF production. If, alternatively, decreased LIF response in PBL cultures is due to circulating predifferentiated suppressor cells, these would of necessity suppress lymphokine production while leaving lymphoproliferation intact.

Mechanism of recurrent disease: a hypothesis. The observation that latent HSV infections appear to persist for the lifetime of the host (4, 6, 9, 12, 21, 30, 34, 38) indicates that the virus avoids complete elimination by the immune system. This could be due to the persistence of the virus as a repressed genome or to its limited replication, i.e., at levels at which it eludes immune detection (30). Whatever the mechanism of virus persistence, it is likely that the first event in the expression of recurrent disease occurs in a microenvironment that is not initially recognizable as non-self to the afferent arm of the immune system. Accordingly, our hypothesis envisions recurrent disease as occurring at a time when the immune effector responses, which are characteristically antigen driven, are minimal or altogether absent. It argues that increased virus replication, accompanied by the expression of cell surface antigens, provides the stimulus necessary for the generation of an anamnestic effector response capable of eliminating infected cells and checking cell-to-cell spread of virus. If prompt and efficient, this response would check virus spread early, conceivably resulting in little or no symptomatology. In contrast, a lag in the generation of the anamnestic response, such as might result from the presence (or the induction) of suppressor cell population(s), would temporarily favor the replication of the virus resulting in clinically apparent recurrent disease due to viral cytopathic effect or immune destruction of infected tissue. This hypothesis thus posits regulatory aspects of virus-specific CMI at the center of the recurrent herpetic disease problem by predicting that the speed and efficacy of the generation of an active CMI effector response from a state of immune memory will determine the clinical outcome of the recurrent event.

Although cause and effect cannot be argued on the basis of our data, particularly since it is difficult to extrapolate from in vitro to in vivo events, they support the hypothesis by demonstrating that recurrent disease is associated with impaired generation of anamnestic CMI responses, as reflected in altered kinetic and dose-response patterns of in vitro secondary responses (Tables 4 and 5). Conceivably, such an impairment could result from decreased numbers of appropriate cell populations, inefficient interaction of immune and accessory cells, or the intervention of active suppression. Experiments designed to distinguish between these alternatives and to determine whether the effects observed in vitro play a decisive role in disease manifestations are currently in progress in our laboratory.

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