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# Native and Recombinant Herpes Simplex Virus Type 1 Envelope Proteins Induce Human Immune T-Lymphocyte Responses

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**The abilities of whole herpes simplex virus type 1 (HSV-1) antigen (HSV-ag) and purified HSV-1 native and recombinant envelope proteins to stimulate in vitro T-lymphocyte responses were compared in patients with recurrent herpes labialis. Immunochemically purified preparations of native glycoproteins B, C, and D (ngB, ngC, ngD) from cultured HSV-1 as well as expressed recombinant plasmid preparations of gD (rgD-1t, rgD-45K) elicited lymphocyte proliferation (LT) and production of gamma interferon (IFN-γ) and interleukin-2 (IL-2) only in seropositive individuals. The IFN-γ induced by rgD-1t correlated with the time to the next herpetic lesion in 19 volunteers followed to recurrence ($r = 0.69$, $P < 0.008$), although the magnitude and frequency of LT and IFN-γ responses were lower with either recombinant or native purified antigens than with the whole-virus antigen. Combinations of ngB plus ngD or ngB plus ngC plus ngD stimulated more IFN-γ, equivalent to whole-virus-antigen responses. Recombinant-derived human IL-2 also specifically increased LT and IFN-γ responses in antigen-driven cultures. ngD stimulated IL-2 and LT responses similar to those of whole-virus antigen and higher than those of ngC. HSV-ag and ngB induced significantly higher titers of total IFN than could be accounted for by IFN-γ; this was not seen for the other antigens, which induced only IFN-γ. HSV-ag-driven Leu $2a^-$, plastic-nonadherent blood cells, unlike whole peripheral blood mononuclear cells, showed evidence of an increase and then a decline in the frequency of HSV-responsive cells after a lesion recurrence. These studies suggest that HSV-1 envelope proteins are capable of stimulating an immune T-helper-cell response which is associated with the prevention of human herpes simplex lesion recurrence. Although the whole virus probably contains additional important antigens, increasing concentrations or combinations of certain purified glycoproteins or the addition of nonspecific enhancers of T-lymphocyte function can drive in vitro immune responses to the same level as the complete set of viral antigens.**

Although human immune responses to herpes simplex virus type 1 (HSV-1) infection involve multiple humoral and cell-mediated mechanisms (8, 24, 26), few have predicted the intervals between recurrent lesions (11, 27). The ability to augment such an immune mechanism may lead to an approach to suppression of the clinical manifestations of reactivation of latent HSV infection. One such response, the titer of gamma interferon (IFN-γ) produced in vitro by HSV-1 antigen (HSV-ag)-stimulated T lymphocytes (primarily the Leu $3^+$, T helper population [9]), has a positive correlation with the ensuing lesion-free interval in individuals with recurrent herpes labialis (RHL) (26, 34). This relationship between high IFN-γ and longer time to lesion recurrence is supported by the observation that adults with this disease have lower HSVag-triggered IFN-γ production than do those with serological evidence of past infection, but no recurrent sores (16). This response in vivo also has direct clinical correlation, as titers of IFN-γ found in lesion vesicle fluid also correlate with time to next lesion recurrence. Alpha and beta interferons (IFN-α and IFN-β), also present in these lesions, have no such correlation (33; J. W. Torseth, B. J. Nickoloff, T. Y. Basham, and T. C. Merigan, J. Infect. Dis., in press).

Which HSV proteins induce IFN-γ and other human T-lymphocyte responses are only partially known (8). However, the recent purification of various viral envelope proteins by immunochemical and recombinant DNA technologies (6, 32, 37) has begun to allow dissection of their antigenic activity (10, 13, 17, 39). Several lines of animal experimentation suggest that the envelope glycoproteins B, C, and D (gB, gC, and gD) have roles in the immune response to HSV. gD induces murine virus-neutralizing antibody (7). In addition, vaccination with gB, gD, or recombinant or synthetic peptides of gD or administration of monoclonal antibody to gB, gC, or gD protects mice from lethal virus challenge (1, 3, 12, 23).

The current study gives further evidence for the importance of Leu $3^+$ T cells in RHL and examines human immune T-lymphocyte transformation and lymphokine responses to purified preparations of three glycoproteins from HSV-1-infected mammalian cells. The responses to these proteins were immune specific and concentration dependent. The response to native gD (ngD) was greater than those induced by either of two recombinant gD polypeptides. In addition, the IFN-γ titers induced by gD correlated with the time to the patients' next lesions. In vitro responses could be augmented by combinations of purified antigens or by the addition of purified recombinant-derived human interleukin-2 (IL-2).

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## MATERIALS AND METHODS

**Population of patients.** Healthy volunteers were seen at Stanford Medical Center after responding to announcements. Those with RHL confirmed by isolation of HSV-1 (fluorescent-antibody staining [Syva, Palo Alto, Calif.] of infected rabbit kidney cells) were bled twice, 7 days apart, to establish macrophage and then nylon wool-nonadherent (NWNA) cell cultures. Seronegative laboratory volunteers had neither a history of HSV infection nor serum antibody to HSV by complement fixation assay.

**Preparation of antigens.** HSV-ag was produced by UV light inactivation of HSV-1 strain MacIntyre grown in Vero cell culture and stored at −70°C as previously described (34). Control antigen was identically prepared from uninfected cultures. The concentration of gD protein in this whole-virus antigen was determined by using a sandwich-type enzyme-linked immunosorbent assay employing type-nonspecific monoclonal antibody against this glycoprotein (rgD-1t was used as a positive control). This antigen had virus titers of $1 \times 10^8$ to $1.5 \times 10^8$ PFU/ml before inactivation.

Immunosorbent chromatographic purification of native gD from cytoplasmic extract of HSV-1 strain NS was performed as described previously for strain HF virus (14). The strain HF preparation, when analyzed by Coomassie blue-stained polyacrylamide gel electrophoresis (PAGE), yielded three protein bands, each of which had virtually identical tryptic peptide profiles. Strain NS (ngD) preparation had strong bands at 58,000 to 60,000 (in a Laemmli-type sodium dodecyl sulfate [SDS]-PAGE [20]) and at 112,000 to 114,000 with two much weaker bands at 47,000 (similar to strain HF gD) and at 55,000 (see Fig. 1). The ngD was dialyzed extensively against 0.01 M Tris-buffered saline, lyophilized, reconstituted in sterile distilled water, and stored in aliquots at −70°C before being freshly thawed for addition to lymphocyte cultures.

The immunosorbent chromatographic purification of ngB and ngC from cytoplasmic extracts of strain NS was similar to that of ngD. Briefly, monoclonal antibodies 24-S (30) and 1C8 (from H. Friedman, Philadelphia, Pa.) were adsorbed to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) following the recommendations of the manufacturer. Solubilized protein extracts of sonicated BHK cells (infected to 100% cytopathic effect by virus) were then incubated on the prewashed columns. After extensive washing, columns were eluted with 3 M KSCN (ethanolamine was used to elute gC), and again eluates were dialyzed extensively against 0.01 M Tris-buffered saline, concentrated, and lyophilized. After reconstitution, storage, and SDS-PAGE as above, the ngB revealed a triplet major band (≥94% of all detected protein by spectrophotometric scanning) at 116,000 to 117,000, while the ngC revealed a strong band at 108,000 to 109,000 (≥76%, molecular weight similar to that ascribed to gC precursor protein [31]) and a faint band at 82,000 to 84,000.

To show that the native glycoproteins purified by immunosorbence were free of cross contamination, purified ngB (25 mg), ngC (30 mg), and ngD (15 mg) were electrophoresed on a 10% nondenaturing SDS-polyacrylamide gel and electroblotted (5). This was done in triplicate, and each panel of three proteins was reacted with rabbit polyclonal antisera prepared to each of the purified glycoproteins, following the method of Cohen et al. (5). The blots were then probed with iodinated protein A. Figure 1b shows that each of the antisera reacted with the corresponding glycoprotein and not with the other two.

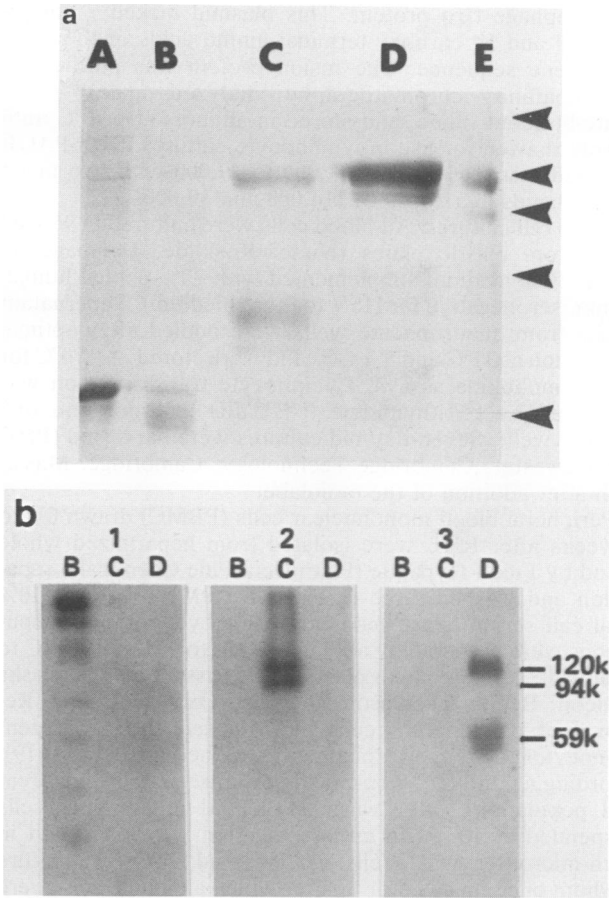


FIG. 1. (a) SDS-PAGE of HSV antigens. Photograph of Coomassie blue-stained Laemmli-type polyacrylamide gel. Lanes: A, rgD-45K; B, rgD-1t; C, ngD; D, ngB; E, ngC. Arrows represent markers at 200,000, 116,000, 97,000, 66,000, 45,000 (in order from the top). (b) Reactivity of rabbit antisera to ngB (1), ngC (2), and ngD (3). Immunosorbent-purified ngB, ngC, and ngD (lanes B, C, and D, respectively, in each panel) were electrophoresed in a native gel (10% acrylamide cross-linked with bisacrylamide) and transferred to nitrocellulose. k, $\times 10^3$.

Recombinant-derived glycoprotein gD-1t (rgD-1t) was obtained from concentrated culture medium conditioned by the growth of the Chinese hamster ovary gD10.2 cell line (21) which expressed a recombinant plasmid (pBR322) containing the gD gene cloned from an HSV-1 clinical isolate (2). The soluble protein was purified by immunoaffinity chromatography and was dialyzed against 0.01 M Tris-buffered saline. It was stored at 4°C until use in cell cultures (less than 4 weeks). SDS-PAGE of rgD-1t revealed a strong band at 45,000 to 50,000 and a faint band at 39,000 to 41,000 (precursor form of gD-1t). The expressed protein contained the amino-terminal 275 amino acids of mature gD (or the $NH_2$-terminal 300 amino acids of the complete gene) (21).

A plasmid similar to pEH25 (38) produced a nonglycosylated truncated form of gD (rgD-45K) used in these experiments. Prepared originally from HSV-1 strain Patton, the cloned gD gene was expressed in *Escherichia coli* as a chimera protein containing 23 amino acids of lambda

bacteriophage Cro protein. This plasmid lacked 7 amino-terminal and 54 carboxy-terminal amino acids of the complete gene sequence. The fusion protein was purified by immunoaffinity chromatography, dialyzed against phosphate-buffered saline, and stored in aliquots at −70°C until freshly thawed for use in lymphocyte cultures. SDS-PAGE revealed a strong band at 43,000 to 49,000 with two much fainter bands at 110,000 to 114,000 and 30,000.

**Blood cell cultures.** All blood cells were cultured in 96-well flat-bottom plastic plates (Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 20% pooled human serum, seronegative for HSV (culture medium). Supernatant media from quadruplicate wells were pooled after optimal incubation at 37°C and 5% $CO_2$ and were stored at −70°C for later lymphokine assays. Lymphocyte transformation was measured by [$^3$H]thymidine ([$^3$H]TdR) incorporation (0.5 μCi per well). Seven-day-old cultures were harvested (PHD cell harvester; Cambridge Technology, Cambridge, Mass.) 18 h after addition of the radiolabel.

Peripheral blood mononuclear cells (PBMC) drawn 0.5 to 4 weeks after RHL were isolated from heparinized whole blood by Ficoll-Hypaque (Pharmacia Fine Chemicals) separation and washed three times with RPMI 1640 with 10% fetal calf serum (wash medium). Monocyte-enriched populations were prepared by adding $3 \times 10^7$ to $4 \times 10^7$ PBMC to fetal calf serum-coated plastic T-75 tissue culture flasks (Falcon; Becton Dickinson Labware, Oxnard, Calif.). Removal of nonadherent cells and, subsequently, adherent monocytes were performed as described previously (9), affording a 60 to 80% nonspecific esterase-staining adherent cell population. After washing, 200 μl of adherent cells suspended at $10^5$/ml in culture medium was inoculated in each microtiter well. Wells were washed with fresh culture medium once on day 5 or 6, after which all viable cells were macrophages as previously described (9) and were stainable with nonspecific esterase.

Fresh PBMC ($3 \times 10^7$ to $4 \times 10^7$ in 3 to 5 ml of wash medium) from the second venipuncture of each volunteer were incubated on a water-jacketed, prewashed, nylon wool column at 37°C (35). Cells were eluted with an optimal volume of wash medium and contained <3% HLA-DR (Becton Dickinson Labware) and <4% surface immunoglobulin-bearing cells (Tago Inc., Burlingame, Calif.) by cytofluorographic analysis. After aspiration of supernatant medium from macrophage-containing wells, $3 \times 10^5$ NWNA cells in 200 μl of fresh culture medium were added to each well, along with 50 μl of appropriately diluted antigen or control (in RPMI 1640). In preliminary experiments, PBMC were seeded alone at $3 \times 10^5$ cells per well with identical volumes of antigen and culture medium. However, observed responses were higher when purified cell populations from these patients were studied; thus, only the NWNA T-lymphocyte results are described. Leu 2a$^-$, plastic-nonadherent cell populations were separated by adherence of Leu 2a (Becton Dickinson) antibody-bound cells to goat anti-mouse immunoglobulin G (Zymed, San Francisco, Calif.)-coated petri dishes, according to a modification of the method of Wysocki and Sato (9). Recombinant IL-2 (Cetus Corp., Emeryville, Calif.) was diluted in RPMI 1640 and added to some wells in a total volume of 10 μl.

**Limiting dilution analysis of responder-cell frequency.** Graded numbers of responder cells from RHL patients were plated in 50 μl in 96-well, round-bottom microtiter plates (Flow Laboratories, Inc., McLean, Va.) (36). Two sets of 24 replicates were plated per cell concentration; one set received optimal concentrations of HSV-1 antigens, and one received control antigen (50 μl each). The responder-cell types analyzed included PBMC and Leu 2a$^-$, plastic-nonadherent cells.

Autologous antigen-presenting cells were prepared by gamma irradiation (5,000 rads) of PBMC and were transferred to each well in 50 μl of culture medium ($2 \times 10^6$ cells per ml). Addition of these cells did not change resultant PBMC responder-cell frequency, but increased the linearity of resultant data by least-squares analysis.

After 10 days of incubation at 37°C and 5% $CO_2$, cultures were labeled for 18 h with [$^3$H]TdR as described above. Responder cells had greater than mean control well counts per minute 3 standard deviations. Data were evaluated by Poisson analysis (18). Differences between frequencies were ascribed significance ($\alpha = 0.05$) by Searle's global test for homogeneity of independent slopes (29).

**Lymphokine assays.** Culture supernatants were assayed for IFN by a plaque reduction assay in 35-mm tissue culture wells with primary human foreskin fibroblast cells and vesicular stomatitis virus challenge, as described previously (26). This assay detects IFN-α, -β, and -γ (Torseth et al., in press).

IFN-γ was measured in clinical samples by a highly specific radioimmunoassay (Centocor, Inc., Malvern, Pa.) which employs a double-sandwich technique with two monoclonal antibodies to human IFN-γ (4, 34).

IL-2 present in culture supernatants was measured by incorporation of [$^3$H]TdR after 3-day quadruplicate culture of the IL-2-dependent cell line CTLL-22, and samples had IL-2 values interpolated from standard curves by the method of Gillis et al. (15). Two lots of recombinant IL-2 (measured blinded, at several concentrations each) gave values within 10% of stated concentrations.

**Statistical methods.** Two-tailed Student's *t* test (both paired sample and means analysis), Fisher's exact test, and Pearson correlation coefficient were calculated. Probabilities, when appropriate, were interpolated from standard tables.

## RESULTS

**Lymphocyte transformation responses.** To assess the ability of human peripheral blood T lymphocytes to respond to HSV glycoproteins, we compared the stimulation indices of NWNA lymphocytes and 7-day-old macrophages cultured with purified native and recombinant DNA-derived HSV-1 antigens (Fig. 1) with the indices of identical cultures incubated with the whole-virus antigen, HSV-ag. All patients with culture-proven RHL had significant proliferative responses to HSV-ag in this cell culture system (Table 1), as well as in cultured PBMC (data not shown).

In the T-lymphocyte cultures, all native glycoproteins stimulated transformation in nearly all RHL patients at the peak response concentration of 10 μg/ml (Table 1). Both cell culture systems also exhibited transformation when incubated with the recombinant-derived gD preparations (rgD-1t, 1 to 20 μg/ml; rgD-45K, 5 to 90 μg/ml), although a lower frequency of patients had stimulation indices > 3.0. This lack of stimulation did not correlate with the length of time between lesion formation and venipuncture, severity of lesion, or patient age (data not shown). The mean stimulation index produced by HSV-ag was not different from that of ngD. Both of these responses, as well as the ngB-induced response, were significantly higher than those induced by ngC ($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively). The stimulation elicited by the recombinant preparations was

TABLE 1. T-lymphocyte transformation response to HSV-1 antigens

| Antigen | Concn (μg/ml) at peak response | cpm[a] | Stimulation index[b] | No. of responders[c]/no. tested |
|---|---|---|---|---|
| HSV-ag | 0.113 (gD) | 11,500 ± 2,600 | 41.2 ± 6.8 | 19/19 |
| ngB | 10.0 | 4,933 ± 1,225 | 25.8 ± 11.8 | 10/11 |
| ngC | 10.0 | 2,895 ± 1,519 | 5.5 ± 1.7 | 9/11 |
| ngD | 10.0 | 11,100 ± 2,700 | 44.2 ± 9.7 | 10/11 |
| rgD-1t | 2.0 | 5,873 ± 3,700 | 12.0 ± 4.0 | 6/15 |
| rgD-45K | 20.0 | 8,168 ± 4,300 | 16.7 ± 5.0 | 15/20 |
| ngB + ngD | 5 + 5 | 9,767 ± 1,845 | 33.5 ± 19.9 | 11/11 |
| ngC + ngD | 5 + 5 | 5,244 ± 1,730 | 15.4 ± 7.2 | 4/6 |
| ngB + ngC + ngD | 3 + 3 + 3 | 9,305 ± 1,869 | 26.2 ± 10.8 | 11/11 |

[a] Mean ± SEM of [$^3$H]TdR incorporation of quadruplicate wells for the number of patients tested.
[b] Mean ± SEM of stimulation indices for all patients tested. Stimulation index = mean counts per minute of antigen-stimulated cultures/mean counts per minute of control cultures.
[c] Responder was defined by stimulation index > 3.0.

less than that elicited by the ngD as well as the whole-virus antigen ($P < 0.01$). In addition, ngD and HSV-ag triggered higher stimulation indices than the combination of ngD and ngC ($P < 0.02$). The length of incubation of these cultures (7 days) was determined to be optimal for the responses of the crude and rgD-45K antigens (data not shown).

In cell cultures prepared from seronegative healthy volunteers, stimulation indices were less than twice control values for HSV-ag (nine volunteers), ngD, ngB, and ngC (five volunteers each), rgD-45K (three volunteers), and rgD-1t (nine volunteers); the mean stimulation indices of seronegative cell cultures were lower than those of seropositive cell cultures for each antigen. In addition, macrophage cultures without lymphocytes showed no or minimal proliferative responses (mean stimulation index, 1.5) when incubated with HSV-ag, indicating that NWNA lymphocytes were the responding cells.

The dose response of antigen-induced [$^3$H]TdR incorporation (mean ± standard error of the mean [SEM]) from four patients is depicted in Fig. 2. The protein concentrations which elicited maximal responses varied between individuals and between antigens. However, cells from each seropositive individual were tested with a range of antigen concentrations which included the apparent optimal concentration. The ngD and HSV-ag antigens had not induced apparent maximal responses at the highest concentrations

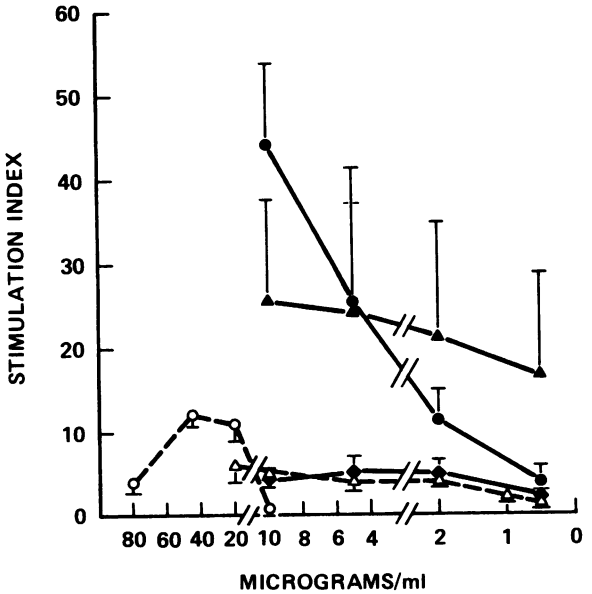


FIG. 2. Proliferative responses of NWNA lymphocytes to HSV-1 antigens from patients with RHL. The curves represent the mean (± SEM) stimulation indices of cultures (from four individuals) incubated for 7 days with various concentrations of ngB (▲), ngC (◆), ngD (●), rgD-1t (△), rgD-45K (○).

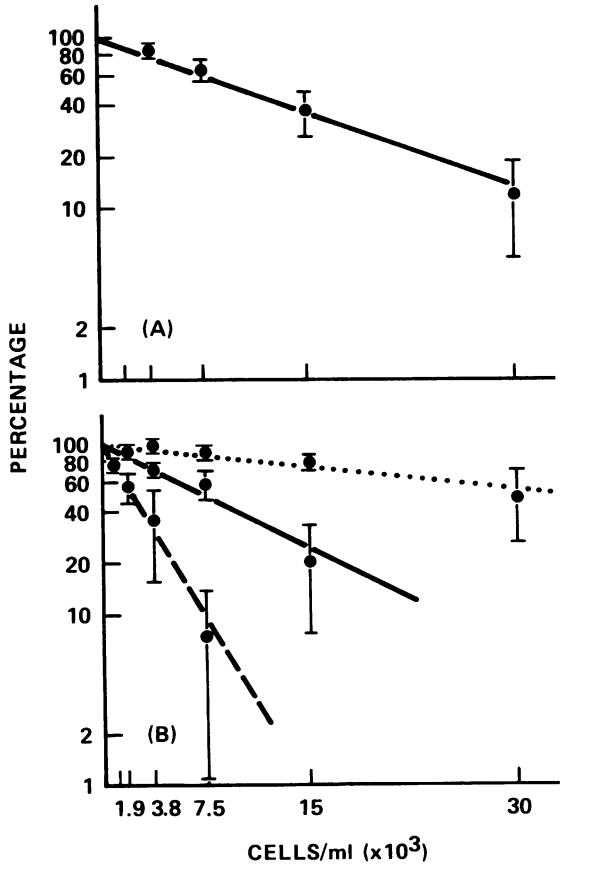


FIG. 3. Limiting dilution analysis of cells responsive to HSV-ag. (A) PBMC nonresponder-cell frequency is plotted versus number of cells seeded per well. (B) Similar plot analyzing Leu 2a$^-$, plastic-nonadherent nonresponder-cell frequency at three time periods after a recurrent oral lesion. Plotted points on the semilogarithmic graph represent means ± SEMs (bars) of at least three determinations. Solid line, 0 to 1 days after onset of lesion erythema; dashed line, 14 to 16 days; dotted line, 42 to 61 days. All determinations were performed at least 24-fold.

TABLE 2. Responder-cell frequencies after RHL

| Responder cell | Time after lesion onset (days) | No. tested | RCF[a] |
|---|---|---|---|
| PBMC | 0–1 | 4 | 1:18,300 ± 2,900 |
| | 7–8 | 4 | 1:17,100 ± 1,900 |
| | 14–15 | 5 | 1:14,600 ± 900 |
| Leu 2a$^-$, plastic nonadherent[b] | 0–1 | 4 | 1:10,200 ± 3,100 |
| | 15–18 | 4 | 1:2,500 ± 1,200 |
| | 42–61 | 4 | 1:47,600 ± 8,200 |

[a] Mean responder cell frequency, determined by limiting dilution, ± SEM.
[b] Primarily lymphocytes (>70% Leu 3$^+$ and Leu 4$^+$, >40% DR$^+$, and <2% Leu 2a$^+$ by cytofluorographic analysis, <2% nonspecific esterase staining). See materials and methods.

used here (0.113 μg of gD per ml for the HSV-ag). These were limited by antigen quantity and total well volume, respectively.

**Limiting dilution analysis of responder-cell frequency.** The frequencies of cells responding to HSV-ag were determined in RHL patients at various times after lesion recurrences. When examined at 0 to 15 days after the onset of a sore, the PBMC responder-cell frequencies of seven patients (Fig. 3a) averaged 1:16,800 ± 4,080 (standard deviation), with no apparent rise or fall with time after lesion (Table 2). However, in four patients followed for a longer period after lesions, Leu 2a$^-$, plastic-nonadherent responder-cell frequencies initially rose and then declined with time after the lesion (Fig. 3b). The differences between each of these three fitted lines are statistically significant ($\alpha \leq 0.05$) by the global test for homogeneity of independent slopes (29). The linear appearance of these plotted data suggest that Poisson analysis employing a single-hit hypothesis was appropriate.

**Lymphokine production.** IFN-γ production by HSV-ag, native glycoproteins, and rgD-45K antigen- triggered macrophage NWNA lymphocyte cultures was measured via radioimmunoassay at various incubation intervals (Fig. 4A). Maximal IFN-γ production by these antigens occurred between days 5 and 8 of culture. Day 6 was subsequently chosen for all remaining culture supernatant harvests. The protein concentrations at which peak IFN-γ production occurred (Fig. 4B) were more sharply defined for the recombinant antigens than their lymphocyte proliferation responses. All three native glycoproteins and the HSV-ag had not reached peak dose response at the maximal culture concentrations used, which were limited as before. The concentration of HSV-ag which induced peak IFN-γ titers had a gD concentration of 0.113 μg/ml ($1.91 \times 10^{-6}$ M). The native glycoprotein concentrations which induced maximal IFN-γ in most patients were 2 to 10 μg/ml. The apparent decline in IFN-γ production occurring at higher recombinant antigen concentrations, also observed with the lymphocyte proliferation response (Fig. 2) could be due to the induction of cell-mediated suppression or to direct suppression by an uncharacterized component(s) in our antigens remaining after purification procedures. However, the titers of IFN-γ induced by purified gD correlated well with the time to next HSV lesion occurrence in patients ($r = 0.62$, $P < 0.008$) (Fig. 5).

Trends toward greater IFN-γ induction by ngD compared with ngB and by ngB compared with ngC did not reach statistical significance (Table 3; $P = 0.10$ for each comparison). HSV-ag stimulated more IFN-γ than each of these proteins ($P \leq 0.05$). However, combinations of ngB plus ngD or ngB plus ngC plus ngD elicited titers indistinguishable from those of the whole-virus antigen ($P < 0.95$ and $P < 0.75$, respectively). Both of these combinations induced more IFN-γ than individual component glycoproteins at identical concentrations ($P \leq 0.04$).

All HSV-ag-triggered cell cultures from individuals with RHL had IFN titers higher than 10 U/ml as measured by plaque-reduction bioassay. Of this total IFN, only 10 to 20% was accounted for by IFN-γ, as measured by radioimmunoassay. However, identical cultures stimulated by the gD antigens and ngC infrequently produced ≥10 U of IFN per ml by bioassay (significantly lower by Fisher exact test, $P \leq 0.03$). Also, in the three cases tested by both assays, all IFn stimulated by these purified antigens was of the gamma type.

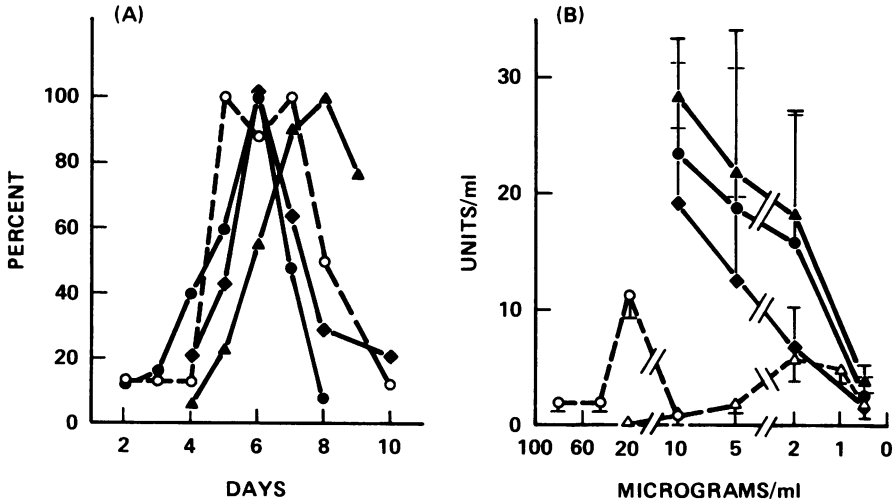


FIG. 4. (A) Kinetics of IFNγ titers in HSV-1 antigen-stimulated NWNA lymphocyte culture supernatants. Time in days after onset of culture incubation are plotted versus percentage of peak IFN-γ titer. Values are representative of four experiments. The following antigens are represented; ngB (▲), ngC (◆), ngD (●), rgD-45K (○). (B) Dose response of IFN-γ titers in HSV-1 antigen-stimulated culture supernatants. The curves represent the means ± SEMs of IFN-γ from four individuals' T-lymphocyte cultures induced by the above-labeled antigens as well as by rgD-1t (△).

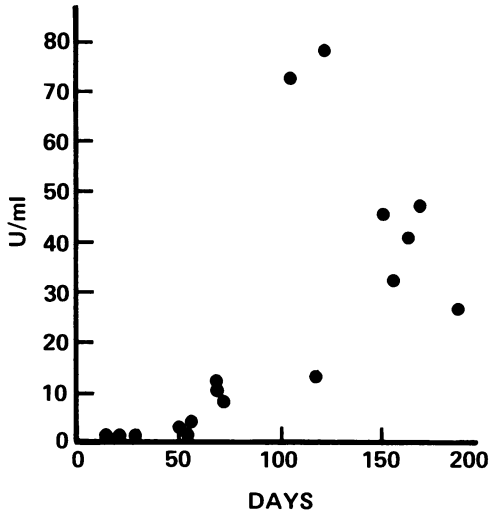


FIG. 5. Relation of purified D-induced titers of IFN-γ produced by nylon-wool purified T-lymphocyte cultures to the time interval until the next recurrence of HSV infection in healthy patients. IFN-γ was measured in pooled supernatants of quadruplicate wells containing $3 \times 10^5$ T cells, 10 μg of gD per ml, and $10^4$ autologous macrophages per well. ●, Patients with documented subsequent recurrence of lesions.

In contrast to this, ngB induced IFN in a pattern similar to that of HSV-ag, presumably stimulating IFN-α or IFN-β or both in addition to the IFN-γ.

IL-2 was induced in 5- to 6-day-old immune NWNA lymphocyte cultures by each antigen. Significantly more was present in supernatants of ngD- and whole-virus antigen-driven cultures than in those stimulated by ngB or ngC ($P \leq 0.05$ for ngD, $P \leq 0.03$ for HSV-ag).

**Augmentation of responses by IL-2.** Low concentrations (1 to 4 U/ml) of recombinant IL-2 added to HSV-ag (0.004 to 0.001 μg of gD per ml)-stimulated cultures induced transformation indices and IFN-γ titers that were higher than the responses to each agent alone (higher even than the sum of these responses; $P < 0.02$, by paired $t$ test [Table 4]). Similar response augmentation was observed, although not to the same degree, when higher concentrations of either stimulant were present, as noted in the higher-dose (20 to 40 U/ml) IL-2 data. The kinetics of optimal IFN-γ production were not affected, except that low titers of IFN-γ (2 to 8 U/ml) appeared by day 1 of HSV-ag-plus-IL-2 culture.

In other experiments, similar enhancements of immune responses were found by the addition of 2 to 5 U of IL-2 per ml to native and recombinant antigen-stimulated cultures (Table 5).

## DISCUSSION

The responder-cell frequency studies reported here show that a circulating population of proliferating, Leu $3^+$-enriched T lymphocytes is induced by a recurrent HSV-1 infection, which increases and then decreases in number with time after the lesion. Removal of plastic-adherent and Leu 2a-expressing cells in our responder-cell frequency assays enriched this population with Leu $3a^+$ cells. These cells are known to proliferate and to produce the majority of immune IFN-γ when induced by HSV-ag (9). Our observations are consistent with previous observations of waxing and waning T-lymphocyte functions (including IFN-γ production) after symptomatic viral reactivation (8, 26). They support the hypothesis that HSV lesions boost systemic cellular immune responses and then recur at a time of lowered immune responsiveness to the virus (8, 19).

Interest in cellular immune responses to HSV-1 antigens has been stimulated by the efficient purification of large quantities of viral proteins which represent potential vaccines (8). Vaccine candidates include viral envelope glycoproteins at least three of which, ngB, ngC, and ngD, stimulate neutralizing antibody formation in animal models (25). A role for glycoprotein-specific, Leu $3^+$ T-lymphocyte-mediated immune responses in host defenses against HSV is supported by the observation that passive transfer of gB-induced helper T lymphocytes to naive mice confers protection from subsequent lethal challenge (3). Our study demonstrates that these HSV-1 glycoproteins as well as truncated recombinant DNA-derived gD induce human T-lymphocyte transformation as well as IL-2 and IFN-γ production in an immune-specific fashion in individuals with culture-proven RHL. These results are consistent with findings of others that certain synthetic gD-1 polypeptides and truncated, recombinant gD-1 and gB-1 proteins induce proliferation (and IL-2 production) by PBMC from seropositive individuals (10, 39). Furthermore, our results support the possible use of a subunit HSV vaccine to boost protective responses in recurrently infected individuals.

In addition, differences between the responses induced by these protein antigens were seen in the present study. ngD and ngB proteins induced higher stimulation indices than ngC, and ngD induced higher IL-2 levels in culture supernatants than gB or gC. A similar pattern was noted for IFN-γ induction by these antigens (gD > gB > gC), although this trend was not statistically significant. However, only a few of these measurements of T-lymphocyte stimulation were in the ranges induced by the whole-virus antigen used for comparison in these studies. This finding, along with the low concentration of glycoprotein present in the whole-virus antigen, suggests that additional antigens exist in the whole virus that are recognized by a significant proportion of the immune-specific T lymphocytes which produce these responses. It will be interesting to compare these findings with the results from the animal vaccination and protection experiments employing these antigens since other gC and gD

TABLE 3. T-lymphocyte immune lymphokine responses[a] to HSV-1 antigens

| Antigen[b] | IFN[c] | | IL-2[c] |
|---|---|---|---|
| | Total IFN | IFN-γ | |
| HSV-ag | 309 ± 157 (25) | 44.9 ± 10.9 (25) | 2.5 ± 0.45 (4) |
| ngB | 127 ± 31 (3) | 20.7 ± 6.2 (11) | 1.5 ± 0.48 (4) |
| ngC | <30 (3) | 14.9 ± 6.8 (11) | 1.0 ± 0.43 (4) |
| ngD | <30 (3) | 23.9 ± 8.9 (15) | 3.1 ± 0.83 (4) |
| rgD-1t | <10 (5) | 6.0 ± 4.1 (19) | |
| rgD-45K | <10 (5) | 10.1 ± 5.2 (20) | |
| ngB + ngD | | 43.5 ± 18.5 (11) | |
| ngC + ngD | | 25.0 ± 16.0 (11) | |
| ngB + ngC + ngD | | 35.4 ± 17.1 (11) | |

[a] Means ± SEM. Numbers in parentheses represent the number of individuals' cultures tested.

[b] Antigen concentrations were those used in Table 1. All comparisons employing the paired Student $t$ test described in the text were made between lymphokine concentrations from supernatants of identical cultures stimulated with different antigens.

[c] Units per milliliter, standardized to National Institutes of Health reference IFNs. IL-2 concentrations are expressed in terms of units of purified recombinant IL-2 (see Materials and Methods).

TABLE 4. Augmentation of HSV-ag-induced T-lymphocyte responses by IL-2

| No. tested | IL-2 concn (U/ml) | Lymphocyte stimulation indices | | | IFN-γ titers | | |
|---|---|---|---|---|---|---|---|
| | | IL-2 alone | HSV-ag alone | Combination | IL-2 alone | HSV-ag alone | Combination |
| Low-dose IL-2 (8) | 1–2 | 3.1 ± 1.4 | 8.4 ± 3.4 | 18.4 ± 4.1[a] | 2.7 ± 1.0 | 4.1 ± 1.6 | 10.6 ± 3.3[a] |
| High-dose IL-2 (6) | 20–40 | 12.6 ± 5.7 | 5.8 ± 1.9 | 14.8 ± 3.1 | 8.8 ± 0.8 | 13.3 ± 3.5 | 25.5 ± 3.1[a] |

[a] Significantly higher response than either IL-2- or HSV-ag-induced responses alone (Student's $t$ test of two means, $P \leq 0.02$).

preparations have been shown to induce murine primary immune T-lymphocyte responses (28).

We also showed that the recombinant antigens are capable of inducing human immune responses that have direct clinical correlation. rgD-1t induced IFN-γ titers which significantly correlated with subsequent time to lesion recurrence in 19 patients. This agrees well with previous observations with native HSV antigens (16, 26, 34). However, the magnitude and frequencies of all immune responses induced by the recombinant antigens were lower than those triggered by the whole-virus antigen, and the LT responses to these antigens were lower than those triggered by ngD. Further study is needed to determine whether these lower responses elicited by the plasmid-derived proteins represent differences in primary or secondary structure of these antigens, additional components present in the antigen preparations, or other factors related to immune T-cell recognition. These differences were not overcome by increasing antigenic dose, however. In addition, these differences did not appear to depend on the presence of oligosaccharides on the amino-terminal two glycosylation sites on the gD antigens, as the unglycosylated *E. coli*-derived rgD-45K had maximal responses that were indistinguishable from the mammalian cell-produced rgD-1t. T-lymphocyte reactivity with these sites has not been excluded, however, as rgD-45K required higher concentrations to induce equivalent responses.

It is interesting to note that the purified, solubilized gD and gC antigens did not induce significant IFN-α or IFN-β production. One previous study found that monoclonal antibodies to gD (unlike monoclonal antibodies to gB or gC) could inhibit virus-infected cells from inducing PBMC to produce IFN-α (22). Our finding that gB induced significant titers of non-gamma IFN suggests that this purified protein contains a specific site(s) which triggers release of this IFN and which is lacking in the other two glycoproteins.

Finally, we observed dose-dependent increases in T-cell-mediated responses specific for virus component antigens which led to a plateau in responses (or even relative inhibition) at the highest antigen concentrations. Also, certain combinations of glycoprotein antigens induced greater immune IFN production than individual antigens used alone. In addition, low doses of recombinant IL-2 (a possible in vivo vaccine adjuvant) when incubated with suboptimal concentrations of HSV antigens selectively enhanced proliferation and lymphokine responses of immune T lymphocytes. Thus, these results demonstrate that T-lymphocyte-mediated, HSV glycoprotein-specific immune responses can be augmented in ways that may have relevance for future vaccine studies.

TABLE 5. Augmentation of immune T-lymphocyte responses to HSV-1 envelope protein antigens by IL-2[a]

| Antigen (5 μg/ml) | No. tested | IFN-γ titers (U/ml, mean ± SEM) | | |
|---|---|---|---|---|
| | | IL-2 alone | Antigen alone | Combination |
| ngB | 3 | 1.8 ± 0.4 | 5.5 ± 1.4 | 24.9 ± 11.0 |
| ngC | 3 | 1.8 ± 0.4 | 2.8 ± 1.1 | 14.6 ± 9.3 |
| ngD | 5 | 2.4 ± 1.2 | 4.0 ± 1.2 | 16.5 ± 4.9 |

[a] Concentration of IL-2 tested was 2 to 5 U/ml.

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## LITERATURE CITED

1. **Balachandran, N., S. Bachetti, and W. E. Rawls.** 1982. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect. Immun. **37**:1132–1137.
2. **Berman, P. W., T. Gregory, D. Crase, and L. A. Lasky.** 1985. Protection of guinea pigs from genital HSV-2 infection by vaccination with cloned HSV-1 glycoprotein D produced in mammalian cells. Science **227**:1490–1492.
3. **Chan, W. L., M. L. Lukig, and F. Y. Liew.** 1985. Helper T cells induced by an immunopurified herpes simplex type 1 (HSV-1) 115K glycoprotein (gB) protect mice against HSV-1 infection. J. Exp. Med. **162**:1304–1318.
4. **Chang, T. W., S. McKinney, V. Liu, P. C. Kung, J. Vilcek, and J. M. Le.** 1984. Use of monoclonal antibodies as sensitive and specific probes for biologically active human gamma inteferon. Proc. Natl. Acad. Sci. USA **81**:5219–5222.
5. **Cohen, G. H., V. J. Isola, S. J. Kuhn, P. W. Berman, and R. J. Eisenberg.** 1986. Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: use of a nondenaturing ("native" gel) system of polyacrylamide gel electrophoresis coupled with Western blotting. J. Virol. **60**:157–166.
6. **Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg.** 1978. Type common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. J. Virol. **27**:172–181.
7. **Cohen, G. H., M. Ponce de Leon, and C. Nichols.** 1972. Isolation of a herpes simplex virus-specific antigenic fraction which stimulates the production of neutralizing antibody. J. Virol. **10**:1021–1025.
8. **Corey, L., and P. G. Spear.** 1986. Infections with herpes simplex viruses. N. Engl. J. Med. **314**:686–691.
9. **Cunningham, A. L., and T. C. Merigan.** 1984. Leu-3 positive T cells produce gamma interferon in patients with recurrent herpes labialis. J. Immunol. **132**:197–202.
10. **De Freitas, E. C., B. Dietschold, and H. Koprowski.** 1985. Human T-lymphocyte response in vitro to synthetic peptides of herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. USA **82**:3425–3429.
11. **Douglas, R. G., Jr., and R. B. Couch.** 1970. A prospective study of chronic herpes simplex infection and recurrent herpes labialis in humans. J. Immunol. **104**:289–295.

12. **Eisenberg, R. J., C. P. Cerini, C. J. Heilman, A. D. Joseph, B. Dietchold, E. Golub, D. Long, M. Ponce de Leon, and G. A. Cohen.** 1985. Synthetic glycoprotein D-related peptides protect mice against herpes simplex virus challenge. J. Virol. **56**:1014–1017.
13. **Eisenberg, R. J., D. Long, M. Ponce de Leon, J. T. Matthews, P. G. Spear, M. G. Gibson, L. A. Lasky, P. W. Berman, E. Golub, and G. H. Cohen.** 1985. Localization of epitopes of herpes simplex virus type 1 glycoprotein D. J. Virol. **53**:634–644.
14. **Eisenberg, R. J., M. Ponce de Leon, L. Pereira, D. Long, and G. H. Cohen.** 1982. Purification of glycoprotein gD of herpes simplex virus types 1 and 2 by use of monoclonal antibody. J. Virol. **41**:1099–1104.
15. **Gilles, S., M. M. Ferm, W. Ou, and K. A. Smith.** 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. **120**:2027–2032.
16. **Green, J. A., P. N. Weiss, T.-J. Yeh, and S. L. Spruance.** 1985. Immune specific interferon production by peripheral mononuclear leukocytes from patients with primary and recurrent orolabial herpes simplex virus infections. J. Med. Virol. **16**:297–305.
17. **Heber-Katz, E., M. Hollosi, B. Dietschold, F. Hudecz, and G. D. Fasman.** 1985. The T cell response to the glycoprotein D of the herpes simplex virus: the significance of antigen conformation. J. Immunol. **135**:1385–1390.
18. **Henry, C., J. Marbrook, D. C. Vann, D. Kodlin, and C. Wofsy.** 1980. Limiting dilution analysis, p. 138–152. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. Freeman Press, San Francisco.
19. **Klein, R. J.** 1982. The pathogenesis of acute, latent and recurrent herpes simplex virus infections. Arch. Virol. **72**:143–168.
20. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lasky, L. A., and D. J. Dowbenko.** 1984. DNA sequence analysis of the type-common glycoprotein D genes of herpes simplex virus types 1 and 2. DNA **3**:23–29.
22. **Lebon, P.** 1985. Inhibition of herpes simplex virus type-1-induced interferon synthesis by monoclonal antibodies against viral glycoprotein D and by lysosomotropic drugs. J. Gen. Virol. **66**:2781–2786.
23. **Long, D., T. Madora, M. Ponce de Leon, G. H. Cohen, P. Montgomery, and R. J. Eisenberg.** 1984. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. Infect. Immun. **37**:762–764.
24. **Lopez, C.** 1985. Natural resistance mechanisms in herpes simplex virus infections, p. 37–68. *In* B. Roizman and C. Lopez (ed.), The herpesviruses: immunobiology and prophylaxis of human herpesvirus infections, vol. 4. Plenum Publishing Corp., New York.
25. **Norrild, B.** 1980. Immunochemistry of herpes simplex virus glycoproteins. Curr. Top. Mirobiol. Immunol. **90**:67–106.
26. **Rasmussen, L. E., G. W. Jordan, D. A. Stevens, and T. C. Merigan.** 1974. Lymphocyte interferon production and transformation after herpes simplex infections in humans. J. Immunol. **112**:728–736.
27. **Rawls, W. E.** 1985. Herpes simplex viruses, p. 527–561. *In* B. Fields (ed.), Virology. Raven Press, New York.
28. **Schrier, R. D., L. I. Pizer, and J. W. Moorehead.** 1983. Type-specific delayed hypersensitivity and protective immunity induced by isolated herpes simplex glycoprotein. J. Immunol. **130**:1413–1418.
29. **Searle, S. R.** 1971. Linear models, p. 75–134. John Wiley & Sons, Inc., New York.
30. **Showalter, S. D., M. Zweig, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins including the immediate-early protein ICP4. Infect. Immun. **34**:684–692.
31. **Spear, P. G.** 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type-1 infected cells. J. Virol. **17**:991–1008.
32. **Spear, P. G.** 1985. Glycoproteins specified by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), The herpes viruses, vol. 3. Plenum Publishing Corp., New York.
33. **Spruance, S. L., J. A. Green, G. Chiu, T.-J. Yeh, G. Wenerstrom, and J. C. Overall.** 1982. Pathogenesis of herpes simplex labialis: correlation of vesicle fluid interferon with lesion age and virus titer. Infect. Immun. **36**:907–910.
34. **Torseth, J. W., and T. C. Merigan.** 1986. Significance of local gamma interferon in recurrent herpes simplex infection. J. Infect. Dis. **153**:979–984.
35. **Valle, M. G., A. M. Bobrove, S. Strober, and T. C. Merigan.** 1975. Immune specific production of interferon by human T cells in combined macrophage-lymphocyte cultures in response to herpes simplex antigen. J. Immunol. **114**:435–441.
36. **van Oehrs, M. H. J., J. Pinkster, and W. P. Zeijlmaker.** 1978. Quantification of antigen-reactive cells among human T lymphocytes. Eur. J. Immunol. **8**:477–484.
37. **Wagner, E. K.** 1985. Individual HSV transcripts: characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), The herpes viruses, vol. 3. Plenum Publishing Corp., New York.
38. **Watson, R. J., J. H. Weis, J. S. Salstrom, and L. W. Enquist.** 1982. Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in *Escherichia coli*. Science **218**:381–384.
39. **Zarling, J. M., P. A. Moran, R. L. Burke, C. Pachl, P. W. Berman, and L. A. Lasky.** 1986. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. IV. Recognition and activation by cloned glycoproteins gB and gD. J. Immunol. **136**:4669–4673.