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Natural infection with herpes simplex virus type 1 (HSV-1) induces humoral and T cell responses to the HSV-1 glycoprotein H:L complex

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The glycoproteins of herpes simplex virus type 1 (HSV-1) are important targets for the immune system in the control of HSV-1 infections. The humoral and T cell responses to the glycoprotein (g)$H_{t(His)}$:gL complex of HSV-1 were studied in seven HSV-1-seropositive and three HSV-1-seronegative healthy adults. In addition, responses to HSV-1 gD_t were determined. As antigens, purified soluble recombinant forms of the $gH_{t(His)}$:gL complex produced by insect cells and of gD_t produced by yeast cells were used. In contrast to seronegative donors, sera of all seropositive donors contained $gH_{t(His)}$:gL-specific IgG. Using peripheral blood (PB) T cells, $gH_{t(His)}$:gL-specific proliferative T cell responses were detected in all seropositive donors. Culture supernatants of PB T cells stimulated with recombinant $gH_{t(His)}$:gL contained high levels of interferon-γ and no detectable interleukin-4, indicating their Th1 phenotype. These results show that naturally acquired HSV-1 infection induces gH:gL-specific humoral and T cell responses.

Herpes simplex viruses (HSV) are the causative agents of localized skin infections of the oral, ocular, neural and genital regions. In persons whose immune function is compromised, severe and often disseminated HSV infections are observed. At least 11 glycoproteins are encoded by HSV-1. Of these, glycoproteins H and L (gH and gL) are present as a heterodimer in the viral envelope and plasma membranes of HSV-1-infected cells (Hutchinson *et al.*, 1992). The HSV-1 gH:gL complex has an essential function in the fusion of the viral envelope with the plasma membrane and in the cell-to-cell spread of virions (Fuller & Lee, 1992; Forrester *et al.*, 1992). The formation of a hetero-dimer between gH and gL is essential for correct folding and processing of gH (Roop *et al.*, 1993).

Of the HSV-1 glycoproteins, gD has been studied most extensively with respect to immune responses and immunological properties. The role of HSV gH:gL as a possible target of the immune system has been studied less extensively. It is known that monoclonal antibodies (MAbs) to the gH:gL complex can inhibit HSV-1 infections *in vitro* (Buckmaster *et al.*, 1984; Showalter *et al.*, 1981). Passive administration of neutralizing antibodies raised against the gH:gL complex protects mice from zosteriform spread of HSV-1 infections (Forrester *et al.*, 1991; Simmons & Nash, 1985). However, immunization studies with recombinant forms of gH and gL (Ghiasi *et al.*, 1992, 1994*a*, *b*) or recombinant vaccinia viruses expressing gH, gL and gH:gL (Browne *et al.*, 1993; Forrester *et al.*, 1991) induced only limited protection. Subsequently, mice immunized with recombinant complexes consisting of truncated gH and full-length gL produced by a mammalian cell line were shown to be protected from a lethal HSV-1 challenge (Peng *et al.*, 1998).

This latter finding encouraged detailed investigations of immune responses to the HSV-1 gH:gL complex in naturally infected humans. In the present study, a soluble purified recombinant form of the HSV-1 gH:gL complex, produced and secreted by insect cells, was used to analyse the humoral and peripheral blood (PB) T cell responses in seven HSV-1-seropositive and three HSV-1-seronegative healthy volunteers. The seven HSV-seropositive donors had been naturally infected by HSV-1 and most likely not by HSV-2, since none of the sera reacted with recombinant gG2 fragments and gG2 peptides (Oda-Ikoma *et al.*, 1998).

The recombinant complex, designated $gH_{t(His)}$:gL, consisted of full-length gL and truncated gH. The gH molecule

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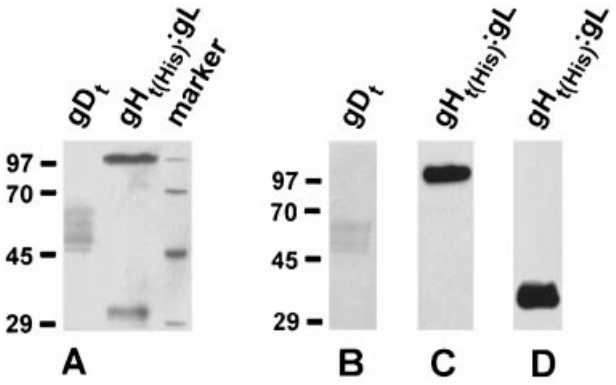

Fig. 1. Recombinant antigens HSV-1 $gH_{t(His)}$:gL and gD_t. Purified $gH_{t(His)}$:gL (A, C, D) and gD_t (A, B) were separated by 12·5% SDS–PAGE. Proteins were visualized by silver staining (A) and by Western blotting (B–D). For Western blotting, the proteins were transferred onto PVDF membranes and subsequently incubated with gD-specific MAb A-16 (B), with gH-specific polyclonal antibody anti-gH1 (rabbit 83) and with gL-specific polyclonal antibody W192S5. Membranes were subsequently incubated with peroxidase-conjugated goat anti-mouse (B) or goat anti-rabbit (C, D) antibodies. Antibody binding was visualized by a luminogenic reaction. Marker proteins with molecular masses of 97, 70, 45 and 29 kDa are indicated.

was truncated before the transmembrane region at amino acid 791 and was tagged with the peptide RSHHHHHH at the C terminus. High Five insect cells (Invitrogen) were infected with recombinant baculoviruses containing the open reading frames of both $gH_{t(His)}$ and gL under control of polyhedrin promoters. After 72 h of infection, the culture medium (Insect Xpress, BioWhittaker) was harvested. The $gH_{t(His)}$:gL complex was isolated in a one-step purification from the culture medium by immobilized metal affinity chromatography (D. F. Westra, unpublished). The purified $gH_{t(His)}$:gL complex was obtained with a high degree of purity, as analysed on SDS–polyacrylamide gels with subsequent silver staining (Fig. 1A). The predicted molecular masses of $gH_{t(His)}$, gL and gD_t are 100, 30 and 47 kDa. Western blots with the appropriate antibodies confirmed the identity of the bands seen on the silver-stained gel (Fig. 1B–D). Size exclusion HPLC (data not shown) showed that the $gH_{t(His)}$:gL complex has a molecular mass of 125 kDa, and confirmed that the complex is a hetero-dimer. The purified $gH_{t(His)}$:gL was recognized by MAb LP11, as assayed by ELISA. Reactivity to LP11 is seen as indicative of correctly folded gH:gL since this antibody recognizes gH only when coexpressed with gL (Hutchinson *et al.*, 1992).

In parallel, the humoral and PB T cell responses to soluble recombinant HSV-1 gD were analysed. The gD_t antigen, truncated at residue 314, was produced in the methylotrophic yeast *Pichia pastoris*. Recombinant gD_t was purified from the yeast medium by a two-step procedure. First, recombinant gD_t was separated by anion exchange chromatography (Resource Q column; Pharmacia) and, subsequently, gD_t was further purified by gel filtration (Superose 6 column; Pharmacia). Recombinant HSV-1 gD_t was seen as at least three diffuse polypeptide bands (Fig. 1), most likely due to heterogeneous glycosylation and proteolysis of gD_t in yeast cell cultures, and was recognized by gD-specific MAbs (data not shown).

The humoral responses to a crude HSV-1 lysate, and to recombinants $gH_{t(His)}$:gL and gD_t were analysed by ELISA. For the preparation of HSV antigen, Vero cells were infected with HSV strain McIntyre (ATCC VR-539) at an m.o.i. of 10. The infected cells were lysed by freeze–thawing followed by heat-inactivation at 65 °C for 1 h. As a control, lysed mock-infected Vero cells were used. The antigens were stored in aliquots at −80 °C. The presence of specific IgG in human sera was analysed by ELISA. A lysate of HSV-1-infected Vero cells and recombinant proteins $gH_{t(His)}$:gL and gD_t were coated overnight in 50 mM sodium bicarbonate buffer pH 9·6. Serial dilutions of the sera were incubated for 1 h. After washing, horseradish peroxidase-conjugated rabbit anti-human IgG antibodies (Dako) were added. The substrate *O*-phenylenediamine.HCl was used and colour development was measured. The antibody levels were defined as the reciprocal of the serum dilution which gave an A_{495} of 0·5.

Seven HSV-1-seropositive individuals (donors 1–7) and three HSV-1-seronegative individuals (donors 8–10) were tested for IgG levels specific for HSV antigen and for $gH_{t(His)}$:gL complex and gD_t (Table 1). Six out of seven HSV-1-seropositive individuals had identical antibody levels to the $gH_{t(His)}$:gL complex. None of the HSV-1-seronegative donors had significant IgG levels to the antigens tested. One seropositive individual (donor 1) had a relatively low antibody level to recombinant $gH_{t(His)}$:gL. This response was, however, still higher than those of the seronegative donors. The antibody levels to gD_t varied to a larger extent. Two donors (donors 5 and 6) had both very high gD_t-specific antibody and very high HSV-specific antibody levels. In general, higher gD_t-specific antibody levels correlated with higher HSV-specific antibody levels. This correlation was not seen for the antibody levels specific for the $gH_{t(His)}$:gL complex. In three out of seven HSV-seropositive individuals, the $gH_{t(His)}$:gL antibody levels were higher than the antibody levels for gD_t (Table 1). The data suggest that the HSV gH:gL complex is an important antigen to which humoral immune responses are elicited upon natural HSV infection.

Peng *et al.* (1998) showed that sera from mice and rabbits which were immunized with correctly folded recombinant HSV-1 gH_t:gL complex had antibodies which neutralized HSV-1 *in vitro*. However, the possible protective role of these neutralizing antibodies *in vivo* was not addressed in this study. Passive administration of an HSV-1 gH:gL-specific MAb has been shown to protect mice from zosteriform HSV-1 infection (Simmons & Nash, 1985). To date, homologues of the gH:gL complex have been found in all herpesviruses investigated. In human donors naturally infected with the herpesvirus human cytomegalovirus (CMV), CMV gH has been identified as a major target antigen of the CMV-specific neutralizing antibodies (Urban *et al.*, 1996). Consequently, HSV-1 gH:gL-

Table 1. Serum IgG antibody levels and PB T cell responses to crude HSV-1/Vero lysate and recombinant proteins HSV-1 $gH_{t(His)}$:gL and gD_t

				[^{3}H]Thymidine incorporation (SI)†							
	Antibody level*			Vero/mock	Vero/HSV-1		Medium	$gH_{t(His)}$:gL		gD_t	
Donor	HSV-1	$gH_{t(His)}$:gL	gD_t	c.p.m.	c.p.m.	SI	c.p.m.	c.p.m.	SI	c.p.m.	SI
1	3000	300	1600	775	41586	54	547	5618	10	33037	60
2	5000	2800	1600	1625	48689	30	260	5599	21	17464	67
3	12000	2800	6000	603	37661	62	370	7690	21	40820	110
4	5500	2600	1600	1170	102821	88	656	17707	27	20672	32
5	80000	2800	19000	1507	45569	30	429	6365	15	5111	23
6	50000	2600	25000	1242	61623	50	379	3544	9	30663	81
7	3500	3200	1600	830	67613	81	721	13613	19	28088	39
8	< 100	< 100	< 100	1721	642	< 1	1 226	600	< 1	2211	2
9	< 100	< 100	< 100	240	829	4	216	2430	11	1225	6
10	< 100	< 100	150	540	296	< 1	567	617	1	1479	3

* Antibody levels were determined by ELISA. Purified recombinants $gH_{t(His)}$:gL and gD_t and lysates of HSV-1-infected Vero cells were used as antigens. The antibody levels were defined as the reciprocal of the serum dilution which gave an A_{495} of 0·5.
† Proliferative responses of 10^5 PBMC against lysates of mock- (Vero/mock) and HSV-1-infected Vero cells (Vero/HSV-1), purified recombinants $gH_{t(His)}$:gL (20 μg/ml), gD_t (20 μg/ml) and medium control (medium) were measured in a 6 day lymphocyte proliferation assay by [^{3}H]thymidine incorporation for the final 16 h. Data presented are means of triplicate cultures in counts per minute (c.p.m.) and the stimulation indices (SI) are given.

Table 2. Concentrations of gamma interferon (IFN-γ) and interleukin 4 (IL-4) in culture supernatants of antigen-stimulated PBMC

Cytokine concentrations (pg/ml) were measured by ELISA in the culture supernatants of PBMC cultures after a 6 day stimulation with a crude lysate of HSV-1-infected Vero cells (Vero/HSV-1), and 20 μg/ml of purified recombinants HSV-1$gH_{t(his)}$:gL and gD_t, respectively. The detection limits of the ELISA for IFN-γ and IL-4 were 5 pg/ml.

	IFN-γ				IL-4			
Donor	Vero/HSV-1	Medium	$gH_{t(His)}$:gL	gD_t	Vero/HSV-1	Medium	$gH_{t(His)}$:gL	gD_t
1	4047	11	3122	3321	< 5	< 5	< 5	< 5
2	4545	12	2875	1249	< 5	< 5	< 5	< 5
3	2556	11	1033	2329	< 5	< 5	< 5	< 5
4	3542	11	2306	1102	< 5	< 5	< 5	< 5
5	2202	12	1053	743	< 5	< 5	< 5	< 5
6	2669	11	830	3252	< 5	< 5	< 5	< 5
7	3789	13	2954	3377	< 5	< 5	< 5	< 5
8	13	12	100	111	< 5	< 5	< 5	< 5
9	322	12	672	161	< 5	< 5	< 5	< 5
10	34	11	20	72	< 5	< 5	< 5	< 5

specific antibodies may have an important role in controlling HSV-1 infection.

Proliferative responses of freshly isolated peripheral blood mononuclear cells (PBMC) to lysates of HSV-1-infected Vero cells and to recombinants $gH_{t(His)}$:gL and gD_t were examined in a lymphocyte proliferation assay. PBMC were isolated by density centrifugation of heparinized PB on a Ficoll gradient (Lymphoprep; Nycomed) and were cultured in RPMI 1640 supplemented with 10% heat-inactivated human serum. 10^5 PBMC per well were incubated with recombinant gD_t (1, 5, 10

and 20 μg/ml), recombinant $gH_{t(His)}$:gL (1, 5, 10 and 20 μg/ml) and lysates of mock-infected and HSV-1-infected Vero cells. T cell proliferation was measured as ^{3}[H]thymidine incorporation over the last 16 h of a 6 day culture period. The stimulation index (SI) for HSV was calculated as the quotient of c.p.m. from cultures stimulated with a lysate of HSV-1-infected Vero cells to c.p.m. from cultures stimulated with a lysate of uninfected Vero cells. The SI for $gH_{t(His)}$:gL and gD_t was calculated as the quotient of c.p.m. from cultures stimulated with the purified glycoproteins to c.p.m. of unstimulated cultures.

PBMC of seven seropositive and three seronegative healthy adult donors were tested on two or more occasions with the antigens. Data from a representative experiment are shown in Table 1. The PB T cells of all HSV-seropositive donors (donors 1–7) responded to lysates of HSV-1-infected Vero cells, recombinant HSV-1 $gH_{t(His)}$:gL and gD_t. Among the three seronegative donors, two donors did not respond to the HSV antigens and donor 9 demonstrated profound PB T cell responses to the two recombinant HSV-1 glycoproteins with only a marginal response to Vero/HSV-1 lysate. The data indicate that following a natural HSV-1 infection, HSV-1 gD but also gH:gL-specific T cell immunity is elicited. Given the nature of the antigen, e.g. exogenous antigen, the antigen-specific T cell responses measured *in vitro* were most likely orchestrated by $CD4^+$ T cells.

T helper cells can be divided into subsets, Th1 and Th2, based on their production of distinct patterns of cytokines. Typical Th1 and Th2 cytokines are interferon (IFN)-γ and interleukin (IL)-4, respectively (Biron, 1994). To determine the Th subset of the antigen-specific PB T cells, the secretion of IFN-γ and IL-4 by antigen-stimulated PB T cells of the HSV-seronegative and -seropositive donors was measured (Table 2). After antigenic stimulation for 6 days, cell-free culture supernatants were assayed for IFN-γ and IL-4 with commercial ELISA kits as described by Verjans *et al.* (1998*b*). PB T cell cultures from donors 1–7 stimulated with HSV-1 lysate, gD_t, and $gH_{t(His)}$:gL secreted large amounts of IFN-γ and no detectable IL-4 (Table 2). Interestingly, PB T cells from donor 9 also secreted significant amounts of IFN-γ upon stimulation with HSV-1 antigen and recombinant $gH_{t(His)}$:gL, and, to a lesser extent, gD_t (Table 2). These results indicated, as described previously, that the HSV-1-specific PB T cell response is Th1-like (Ghiasi *et al.*, 1992; Biron, 1994; Carmack *et al.*, 1996; Cher & Mosmann, 1987; Hendricks *et al.*, 1992).

The HSV-1 glycoproteins gB, gC and gD have been identified as major targets for cytolytic HSV-1-specific $CD4^+$ T cells (Mikloska & Cunningham, 1998). In that study, HLA-DR-expressing human epidermal keratinocytes, infected with recombinant vaccinia virus expressing gB, gC, gD or gH, were used as target cells. The highest cytotoxicity was measured for gD, followed by gB or gC, and then by gH. Beninga *et al.* (1995) analysed the T cell responses to CMV; three out of five CMV-specific T cell lines, mainly consisting of $CD4^+CD8^-$ T cells, responded to CMV gH (Beninga *et al.*, 1996). Our study indicates that naturally acquired HSV-1 infection elicits a gH:gL-specific Th1 response. All HSV-seropositive donors responded to $gH_{t(His)}$:gL in the proliferation assay. Two of the three seronegative donors were negative. The result of the proliferation studies with the PB T cells of HSV-seronegative donor 9 is puzzling. Despite the PB T cell response against gD_t and $gH_{t(His)}$:gL complex, the donor was still HSV-seronegative 1 year after the proliferation assays. The proliferative response to $gH_{t(His)}$:gL could have been caused by PB T cells which recognize peptides of the gH:gL complex of other human herpesviruses. Parts of the gH:gL complex are conserved among herpesviruses. However, the gD_t-specific proliferative response cannot be explained by such a homology because, except for HSV-2, there are no homologues of gD in other human herpesviruses. We cannot exclude the possibility that, despite the absence of contaminating proteins present on the silver-stained SDS-gel, the relatively low PB T responses of donor 9 to $gH_{t(His)}$:gL and gD_t might be directed against contaminating proteins present in very low amounts in the antigen preparations.

The role of T cell immunity in controlling systemic and local HSV-1 infections has been studied extensively. HSV-specific $CD4^+$ and $CD8^+$ cells with cytotoxic and/or proliferating activities are present in high frequencies in PB cells and herpetic lesions (Verjans *et al.*, 1998*a*, *b*; Schmid, 1988; Posavad *et al.*, 1996; Carmack *et al.*, 1996; Koelle *et al.*, 1994). The role of $CD8^+$ T cells may be limited because HSV-infected cells have a reduced surface expression of MHC class I molecules (Hill *et al.*, 1995; York *et al.*, 1994). Nonetheless, $CD8^+$ lymphocytes may have an important function in later stages of herpetic lesions. This is because IFN-γ secreted by stimulated $CD4^+$ Th1 cells upregulates MHC class I expression on HSV-infected cells (Posavad *et al.*, 1998). Alternatively, some cells may be less susceptible to the HSV-induced downregulation of MHC class I (Posavad *et al.*, 1996).

The observation that a natural HSV-1 infection elicits both a T cell (Th1-like) and humoral response to the HSV-1 gH:gL-complex may be of importance for the development of a subunit vaccine. Taking into account the finding that immunization with soluble recombinant HSV-1 gH:gL protects mice from a lethal HSV-1 challenge (Peng *et al.*, 1998), the soluble gH:gL complex may be an important candidate as a component of an effective subunit vaccine for the prevention and/or control of HSV infections.

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