T-cell recognition of the secreted and membrane-bound portions of the herpes simplex virus type 2 (HSV-2) glycoprotein G (sgG-2 and mgG-2, respectively) was compared in symptomatic and asymptomatic HSV-2-infected individuals and in HSV-2-seronegative controls and the responses with HSV-1 glycoproteins C and E (gC-1 and gE-1) were compared. CD4+ T cells from HSV-2-infected individuals specifically recognized both sgG-2 and mgG-2, whereas HSV-1-infected and HSV-seronegative controls did not respond to these glycoproteins. The responses to gC-1 and gE-1, on the other hand, were not type specific, as blood mononuclear cells from both HSV-1- and HSV-2-infected individuals responded in vitro. There was an association between the status of the infection (symptomatic versus asymptomatic) and the CD4+ T-cell responsiveness. Symptomatic HSV-2-seropositive individuals responded with significantly lower Th1 cytokine production to sgG-2 and mgG-2 than did asymptomatic HSV-2-infected carriers, especially within the HSV-1-negative cohort. No differences in T-cell proliferation were observed between asymptomatic and symptomatic individuals. The results have implications for studies of HSV-2-specific CD4+ T-cell reactivity in general and for analysis of immunological differences between asymptomatic and symptomatic individuals in particular.

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects the human genital tract mucosa and is the most common cause of genital ulcer disease in humans (Ahmed et al., 2003; Kinghorn, 1994). The prevalence of HSV-2 antibodies varies depending on the population studied. In the 1990s, 16–33% of pregnant Swedish women were found to be HSV-2 seropositive (Forsgren et al., 1994; Persson et al., 1995). Most individuals infected with HSV-2 are asymptomatic (Koutsy et al., 1990). However, after information and counselling, some ‘asymptomatic’ individuals may become aware of having recurrent genital symptoms corresponding to herpetic infection (unrecognized symptomatic genital herpes), although 20–30% of HSV-2-seropositive individuals seem to remain without symptoms (Frenkel et al., 1993; Langenberg et al., 1989). Both symptomatically and asymptomatically infected individuals shed virus and can thus transmit the disease (Koelle & Wald, 2000; Wald et al., 2000). Overall, HSV-2 is shed for 1–3 days per month (Krone et al., 2000; Wald et al., 1997, 2002) and in 33–68% of cases this shedding occurs in the absence of concomitant lesions (Krone et al., 2000; Wald et al., 1995).

It is not known why genital HSV-2 infection is asymptomatic in some individuals and symptomatic in others, or why the frequency and severity of recurrences vary among symptomatic patients. Prior HSV-1 infection does not protect against HSV-2 infection, although it has been shown to increase the likelihood of asymptomatic HSV-2 seroconversion by a factor of 2·6 (Langenberg et al., 1999). Other factors known to enhance both the incidence and the severity of HSV-mediated disease, as well as the frequency of virus shedding, are the lack of functional NK cells (Biron et al., 1989; Ching & Lopez, 1979) and reduced numbers of CD4+ T cells, as seen among patients with AIDS (Augenbraun et al., 1995; Koelle & Wald, 2000; Posavad et al., 1997; Schacker et al., 1998; Siegal et al., 1981; van Benthem et al., 2001). Furthermore, subjects suffering from immunosuppression or trauma or suffering from other diseases that affect immunological responses are at risk of clinical manifestations of HSV-2 infection and have a greater likelihood of recurrent infections.
from frequent HSV recrudescences have an impaired lymphoproliferative response to HSV-2 during their acute episodes of recurrent illness (Cauda et al., 1989).

CD4+ T cells appear to be especially important in controlling genital HSV-2 infection. Even though clearance of HSV-2 from recurrent genital lesions correlates with the infiltration of both HSV-2-specific CD4+ and CD8+ cytotoxic T cells (Koelle et al., 1998b), the CD4+ T cells infiltrate first and are associated, time-wise, with the drop in infectious virus titre within the lesion (Cunningham et al., 1985; Spruance et al., 1977). Furthermore, CD8-deficient humans do not suffer from any abnormal viral infections, including those caused by members of the family of Herpesviridae, whereas CD4-deficient AIDS patients do (de la Calle-Martin et al., 2001; de la Salle et al., 1999).

Studies in mice confirm these observations, as CD8+/− but not CD4−/− animals can be successfully vaccinated against the disease (Harandi et al., 2001). CD4+ T-cell responses to HSV-2 appear to be directed against envelope glycoproteins, capsid proteins and regulatory elements within the tegument (Carmack et al., 1996; Koelle et al., 1994, 1998a, 2000a, b).

HSV-2 encodes 11 different glycoproteins. Glycoprotein G (gG) is the least conserved protein between HSV-2 and the closely related HSV-1, with a sequence similarity of less than 30%. HSV-2 gG (gG-2) is the only HSV envelope protein to be cleaved post-translationally during processing (Fig. 1). The high-mannose precursor protein has been proposed to be cleaved between the amino acids Arg-321 and Ala-322, as well as between Arg-342 and Leu-343, where both sites are necessary for correct cleavage (R. L. Courtney, personal communication). These events generate a secreted amino-terminal protein (sgG-2) and a carboxy-terminal high-mannose intermediate that is further processed by O-glycosylation to constitute the cell membrane-anchored mature gG-2 (mgG-2). The first 22 amino acids of the sgG-2 protein comprise a signal sequence and are cleaved off before secretion (Liljeqvist et al., 1999). HSV-1 gG (gG-1), in comparison, is much smaller and, similarly to mgG-2, is inserted into the viral envelope (Balachandran & Hutt-Fletcher, 1985; Dall'Olio et al., 1987; Marsden et al., 1984; Roizman et al., 1984; Su et al., 1987). A unique property among the HSV-2 glycoproteins is that both sgG-2 and mgG-2 elicit a type-specific antibody response and can thus be used to distinguish serologically between HSV-1 and HSV-2 infections (Görander et al., 2003; Lee et al., 1985; Svenerholm et al., 1984). Furthermore, T cells from HSV-2-infected individuals have been shown to respond significantly more strongly to mgG-2 than do T cells from HSV-1-infected individuals, whereas the T-cell responses to more conserved proteins such as gB-2 and gD-2 were similar in HSV-1- and HSV-2-infected subjects (Carmack et al., 1996).

We have compared the T-cell proliferative response to purified sgG-2, mgG-2, gC-1 and gE-1 in asymptomatic and symptomatic HSV-2-infected individuals. We found that both sgG-2 and mgG-2 are truly HSV-2 type specific with respect to T-cell proliferative responses. Furthermore, Th1 cytokine responses to sgG-2 and mgG-2 were suppressed in symptomatic patients compared with individuals with asymptomatic HSV-2 infection. T-cell responses to the more conserved proteins, gC and gE, were not type specific. However, symptomatic patients with a concomitant HSV-1 infection could be distinguished through their enhanced T-cell response to both these proteins.

**METHODS**

**Subjects**

HSV-2-seropositive patients were recruited to the study from the STD clinics at Sahlgrenska University Hospital and Uddevalla Hospital. Asymptomatic as well as symptomatic patients were asked about their history of labial herpes indicating HSV-1 infection. HSV-2 infection was confirmed serologically by Western blotting (see below) and HSV-1 infection was assessed serologically using an HSV-1-specific ELISA (see below). The Ethics Committee at the Faculty of Medicine, Göteborg University, approved these studies.

**Symptomatic HSV-2 infections.** Fifty patients (31 females and 19 males, age range 23–79, median 37 years) had a typical history of recurrent genital herpes with more than six episodes per year and 15 of these received daily antiviral therapy to suppress their infection. The duration of antiviral treatment ranged from 6 months to 10 years (median 20 months).

**Asymptomatic HSV-2 infections.** Twenty-two patients (11 females and 11 males, age range 24–66, median 38 years) were recruited from an ongoing screening study of HSV-2 infection in first visitors to the STD clinics and among partners of HSV-2-infected patients. All had been given thorough information about the clinical spectrum.
of herpes and interviewed about genital symptoms. Seropositive patients who, after further information, admitted to having genital symptoms were excluded from the study.

**HSV-2-seronegative controls.** Thirteen individuals (9 females, 4 males) who were seronegative for HSV-2 were recruited from the staff at the Department of Medical Microbiology & Immunology, Göteborg University.

**Western blotting for detection of HSV-2-specific antibodies.** Lysates of HSV-2 (strain B4327/PUR)-infected HEp-2 cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membrane strips were washed in 0·3% TWEEN 20 diluted in Tris-buffered saline, pH 7·5 (TBS-Tween) for 30 min at 37 °C and blocked with 1 ml 3% powdered milk and 4% fetal calf serum diluted in TBS (block buffer) for an additional 30 min at room temperature. Sera (10 μl) were added to each strip and incubated for 2 h. The strips were washed three times in TBS-Tween and HRP-labelled anti-human IgG (Dako) diluted 1:100 in block buffer was added for 1 h at room temperature. After washing twice in TBS-Tween and once in TBS, the strips were developed. The substrate solution consisted of 30 μl H2O2 diluted in 50 ml TBS and 30 mg HRP substrate diluted in 20 ml methanol. Development was stopped in Super Q water. A serum was considered to contain anti-HSV-2 antibodies if there was a reaction to one or more of three bands, representing mgG-2, the high-mannose precursor gG-2 or the carboxy-terminal intermediate (Fig. 1) (Liljeqvist et al., 2002).

**ELISAs.** Plasma was screened for antibodies to HSV-1 with an HSV-1 kit according to the manufacturer’s manual (HerpeSelect ELISA IgG; FOCUS Technologies), and for sgG-2- and mgG-2-specific antibodies using an ELISA as described previously (Görander et al., 2003; Liljeqvist et al., 1998).

**Proteins**

**HSV-2 gg.** sgG-2 was purified from the medium of virus-infected GMK-AH1 cells by immunoadfinity chromatography using the anti-sgG-2 monoclonal antibody (mAb) 4.A5.A9 as described previously (Liljeqvist et al., 2002). For production of mgG-2, 4 mg anti-mgG-2 mAb O1.C5.B2 (Liljeqvist et al., 1998) was coupled to a cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia) column according to the manufacturer’s instructions. A lysate of virus-infected GMK-AH1 cells (HSV-2 strain 333) was harvested and solubilized in TBS containing 1% sodium deoxycholate and 1% NP-40, followed by centrifugation at 2000 g for 10 min and ultracentrifugation at 100 000 g for 1 h. The supernatant was added to the column and recirculated for 1 h, then washed using TBS and 0·5 M NaCl. The proteins were eluted with 0·1 M glycine/HCl buffer (pH 2·8) and neutralized with Tris/HCl (pH 8). The protein concentration was measured by DC ProteinAssay (Bio-Rad).

**HSV-1 gc and ge.** Preparations of affinity-purified gc-1 and ge-1 were produced as described previously (Trybula et al., 2000). GMK-AH1 cells infected with HSV-1 strain KOS 321 were lysed with cold 0·02 M Tris/HCl buffer (pH 7·5) containing 1% sodium deoxycholate, 1% NP-40, 2 mM EDTA and 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride. The mixture was homogenized with several strokes of a Dounce homogenizer and kept on ice for 1 h. The unsolubilized material was pelleted by centrifugation at 130 000 g for 1 h. For gc-1 purification, the supernatant was pre-adsorbed on an immunosorbsent column containing an anti-ge-1 antibody and passed over a column containing an anti-gc-1 mAb (Bergstrom et al., 1992). For ge-1 purification, the supernatant was pre-adsorbed on an immunosorbsent column containing the anti-gc-1 antibody and passed over a column containing the anti-ge-1 mAb. The columns were washed with 0·02 M Tris/HCl (pH 7·5) containing 0·1% NP-40, 0·05 M NaCl and 2 mM EDTA and then without detergent. The adsorbed material was eluted with 0·1 M glycine/HCl (pH 2·4) and immediately neutralized with 1 M Tris/HCl (pH 8·0). The material was centrifuged to near dryness over a microcentrifugal concentrator with a 30 kDa cut-off (PallGelman Sciences), resuspended in PBS and centrifuged again. The final product was suspended in a small volume of PBS and stored at −20 °C. Protein concentration was determined according to a standard Lowry method (DC protein assay kit; Bio-Rad) and purity was assessed by gel electrophoresis.

**HSV-2 antigen preparation.** HSV-2 strain 333 was grown in GMK-AH1 cell monolayers. Virus was recovered from the cell culture after one cycle of freeze–thawing followed by centrifugation to remove cellular debris. Virus preparations containing 2 × 10⁵ p.f.u. ml⁻¹ were inactivated by UV light for 30 min.

**Lymphocyte proliferation assay.** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood using standard density centrifugation on Ficoll-Hypaque (Pharmacia). Freshly isolated PBMC were resuspended in complete medium (see below) and dispersed in flat-bottomed 96-microwell plates (Nunc) at 10⁵ cells well⁻¹ in well 0·2 ml Iscove’s medium supplemented with 10% fetal bovine serum (Biological Industries), 3 μg l-glutamine ml⁻¹ (Gibco) and 0·1 mg gentamicin sulfate ml⁻¹ (Essex LakeMed AB) in the presence or absence of purified sgG-2, mgG-2, gC-1 and ge-1 (all at 1 μg ml⁻¹). UV-inactivated HSV-2 virions (corresponding to 4 × 10⁵ p.f.u. ml⁻¹) or phytohaemagglutinin (PHA; 2-5 μg ml⁻¹). In some experiments, CD4⁺ or CD8⁺ T cells were removed using magnetic beads (Dynal) according to the manufacturer’s instructions. Cells were incubated for 5 days at 37 °C in a humid atmosphere with 7·5% CO₂. After 2 days, 50 μl culture supernatant was collected from each well and frozen at −20 °C until assayed for cytokine content. Six to eight hours before the end of the culture period, 20 μl culture medium containing 1 μCi [6-³H]thymidine (Amersham) was added to each well. The harvesting and subsequent measurement of incorporated radioactivity were performed on an automated filter cell harvester and an argon-activated β-scintillator counter (Inotech). Data were expressed as the arithmetic mean stimulation index (SI), defined as [³H]thymidine incorporated into antigen-stimulated cultures (mean from duplicates) divided by the mean incorporation of corresponding unstimulated control cultures. SI > 3-0 was referred as a positive response.

**Cytokine analysis.** Cytokine measurements on cell culture supernatants were performed using the human Th1/Th2 Cytokine Cytometric Bead Array kit (BD Biosciences Pharmingen) (i.e. IFN-γ, TNF-α, IL2, IL4, IL6 and IL10), according to the manufacturer’s instructions. Cytokine-bound cyometric beads were analysed on a FACS Calibur flow cytometer. Data were analysed using the BD CBA software (BD Biosciences Pharmingen).

**Statistical analysis.** Statistical analyses were done using Student’s t-test.

**RESULTS**

**Proliferative T-cell responses to sgG-2, mgG-2, gc-1 and ge-1 in HSV-2-infected individuals**

We compared the T-cell proliferative response in HSV-2-infected individuals with that of HSV-2-seronegative controls in response to purified HSV glycoproteins sgG-2, mgG-2, gc-1 and ge-1, as well as to whole UV-inactivated HSV-2 virions. To analyse the degree of anti-HSV-1 antibody cross-reactivity in these in vitro responses, we further subdivided our study population into HSV-1-positive and HSV-1-negative individuals based on HSV-1
type-specific serology. Forty-five per cent of the HSV-2-infected individuals and 54% of the HSV-2-seronegative controls were seropositive for HSV-1 antibodies. As seen in Fig. 2(a), HSV-2-infected individuals, but not HSV-1-infected or HSV-seronegative individuals, responded to both sgG-2 and mgG-2, irrespective of their HSV-1 co-infection status, indicating that the in vitro proliferative response to both sgG-2 and mgG-2 is indeed HSV-2 specific. The responses to gC-1 and gE-1, on the other hand, were equally strong in individuals infected with either HSV-1 alone or HSV-2 alone, implying that the T-cell responses to these two proteins are not HSV-subtype specific. Within the HSV-2-seronegative group, the responses to gC-1, gE-1 and whole HSV-2 were restricted to those infected with HSV-1 (Fig. 2a).

To ascertain that CD4+ T cells mediated the proliferative responses measured, we removed either CD4+ or CD8+ cells from the PBMC suspension. As seen in Fig. 2(b), removal of CD4+ cells completely abolished HSV-2-specific proliferation, whereas removal of CD8+ cells enhanced the responses. The latter result was most likely due to an increased proportion of CD4+ T cells in the CD8-depleted PBMC population.

To ascertain that ongoing antiviral treatment did not influence the T-cell proliferative responses, we compared the responses to whole HSV-2 and to concanavalin A and PHA in patients with and without acyclovir treatment. There were no differences in the response to either specific antigen or to mitogen between treated and untreated patients (data not shown).

Finally, we investigated the degree of correlation between the T-cell proliferative responses to the different HSV antigens. There was a relatively high correlation between the responses to the two gG-2 proteins, sgG-2 and mgG-2 ($r = 0.77$). The same held true for the responses to the two HSV-1 proteins, gC and gE ($r = 0.81$). The cross correlations between the HSV-1 and HSV-2 antigens tested were much lower (not shown).

**Proliferative T-cell responses to HSV envelope glycoproteins in asymptomatic versus symptomatic HSV-2-infected individuals**

Next we analysed whether there were any differences in the T-cell response to HSV glycoproteins between asymptomatic and symptomatic HSV-2-infected individuals. There were no differences in the magnitude of the responses to either mgG-2 or sgG-2 between asymptomatic and symptomatic responders (Fig. 3a, b). Furthermore, HSV-1 co-infection, which occurred in 44% of the symptomatic patients and 45% of the asymptomatic HSV-2 carriers, did not have any significant impact on either the incidence or the magnitude of the gG-2-specific T-cell responses (not shown).

We also investigated the T-cell proliferative responses to the HSV-1 antigens gC and gE. The magnitude of the gC-1-specific response differed considerably between asymptomatic and symptomatic HSV-2-infected individuals. As shown in Fig. 3(c), these differences were attributed to a significantly enhanced gC-1-specific response in the symptomatic HSV-2-infected individuals with a concomitant HSV-1 co-infection. Thus, the response to gC-1 was statistically significantly higher in symptomatic compared with asymptomatic HSV-2/HSV-1 co-infected individuals ($P < 0.05$, Fig. 3c). Asymptomatic HSV-2/HSV-1 co-infected individuals had a gC-1-specific T-cell response of the same order of magnitude as the HSV-1-negative population. Among the HSV-2-positive and HSV-1-negative population, there were no differences between asymptomatic and symptomatic individuals in their T-cell response to gC-1 (Fig. 3c).

The responses to gE-1 overall were weaker than the responses to gC-1, but the distribution between HSV-2/HSV-1 co-infected and HSV-1-seronegative individuals was similar to that obtained in response to gC-1. Thus, symptomatic HSV-2/HSV-1 co-infected individuals showed a significantly higher T-cell proliferative response to gE-1 compared with both asymptomatic HSV-2/HSV-1 co-infected (filled bars, $n = 31$), HSV-1-negative HSV-2-infected (hatched bars, $n = 37$), HSV-1-infected HSV-2-negative (open bars, $n = 7$) and HSV-1/HSV-2-negative (shaded bars, $n = 6$) individuals. (b) T-cell proliferative responses to HSV-2 in PBMC depleted of either CD4+ or CD8+ cells. Data are expressed as SI (mean ± SD) ($n = 4$).
We analysed the levels of IFN-\( \gamma \) in asymptomatic and symptomatic HSV-2-infected individuals. The corresponding values for gC-1 were 18 ± 2 for HSV-1-negative and 50 ± 2 for HSV-1-positive individuals, we found that the asymptomatic HSV-1-negative group was responsible for the higher Th1 cytokine production. As shown in Fig. 4, the asymptomatic HSV-1-negative carriers had significantly higher levels of IFN-\( \gamma \), TNF-\( \alpha \) and IL2 in response to sgG-2, mgG-2 and HSV-2. The Th1 cytokine responses correlated well with the proliferative responses within the asymptomatic group, but not in the symptomatic group, as shown for the responses to sgG-2 in Fig. 5. Thus, among the asymptomatic HSV-2 carriers, we found that the levels of IFN-\( \gamma \), IL2 and TNF-\( \alpha \) produced increased proportionally with increased T-cell proliferation (Fig. 5a). In contrast, the levels of IFN-\( \gamma \), IL2 and TNF-\( \alpha \) remained low among symptomatic HSV-2 patients, irrespective of the magnitude of the proliferative responses (Fig. 5b). The ability to produce Th1 cytokines appeared to be similar in asymptomatic and symptomatic individuals, as the levels of Th1 cytokines produced in response to PHA were similar (Fig. 4d).

The cytokine responses to gC-1 and gE-1 did not vary between asymptomatic and symptomatic HSV-2-infected individuals (not shown). Furthermore, the levels of IL4 and IL10 were low overall (not shown). The levels of IL6 ranged from 4 to 10,000 pg ml\(^{-1}\), but there were no differences between the asymptomatic and symptomatic groups for either of the antigens tested (not shown).

The Th1 cytokine responses varied within the asymptomatic group of HSV-2-infected individuals (not shown). Eight of the 21 asymptomatic individuals examined produced high levels of Th1 cytokines, whereas the other 13 produced levels that were similar to those obtained in the symptomatic group. We have no explanation for this heterogeneity. There was no correlation with HSV-1 co-infection or with their antibody responses to sgG-2 or mgG-2. However, these eight individuals had a statistically higher proliferative response to all the HSV antigens tested (compared with the other 13 asymptomatic individuals), but not to PHA. Furthermore, they also produced higher levels of gG-2-specific IL10.

### Antibody responses to gG

Prior HSV-1 infection has been shown to increase the incidence of asymptomatic HSV-2 seroconversion (Langenberg et al., 1999). We found no significant difference in the frequency of HSV-1 co-infection between symptomatic and asymptomatic HSV-2-infected individuals. Forty-four per cent of symptomatic and 45% of asymptomatic HSV-2-infected individuals were HSV-1 co-infected. HSV-1/HSV-2 co-infected individuals presented similar anti-sgG-2 and anti-mgG-2 titres to individuals infected with HSV-2 alone. The titres were not influenced by HSV-2

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**Fig. 3.** T-cell proliferative responses to HSV glycoproteins in asymptomatic and symptomatic HSV-2-infected individuals in relation to HSV-1 co-infection status. Data are expressed as SI (mean ± SD) of symptomatic (filled bars, n = 21 for HSV-1-positive and n = 26 for HSV-1-negative) versus asymptomatic (open bars, n = 10 for HSV-1-positive and n = 11 for HSV-1-negative) HSV-2-infected individuals with or without concomitant HSV-1 infection in response to sgG-2 (a), mgG-2 (b), gC-1 (c) and gE-1 (d). *P* < 0.05.

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**Cytokine responses to sgG-2 and mgG-2 in asymptomatic and symptomatic HSV-2-infected individuals**

We analysed the levels of IFN-\( \gamma \), IL2, TNF-\( \alpha \), IL4, IL6 and IL10 in 48 h cell culture supernatants of PBMC stimulated with purified sgG-2, mgG-2, gC-1, gE-1 and irradiated HSV-2 and compared the cytokine profiles between asymptomatic and symptomatic HSV-2-infected individuals. Statistically significantly higher levels of Th1 cytokines were produced by the asymptomatic patients in response to sgG-2, mgG-2 and whole irradiated HSV-2 (not shown). When our subjects were subdivided into HSV-1-negative and HSV-1-positive individuals, we found that the asymptomatic HSV-1-negative group was responsible for the higher Th1 cytokine production. As shown in Fig. 4, the asymptomatic HSV-1-negative carriers had significantly higher levels of IFN-\( \gamma \), TNF-\( \alpha \) and IL2 in response to sgG-2, mgG-2 and HSV-2. The Th1 cytokine responses correlated well with the proliferative responses within the asymptomatic group, but not in the symptomatic group, as shown for the responses to sgG-2 in Fig. 5. Thus, among the asymptomatic HSV-2 carriers, we found that the levels of IFN-\( \gamma \), IL2 and TNF-\( \alpha \) produced increased proportionally with increased T-cell proliferation (Fig. 5a). In contrast, the levels of IFN-\( \gamma \), IL2 and TNF-\( \alpha \) remained low among symptomatic HSV-2 patients, irrespective of the magnitude of the proliferative responses (Fig. 5b). The ability to produce Th1 cytokines appeared to be similar in asymptomatic and symptomatic individuals, as the levels of Th1 cytokines produced in response to PHA were similar (Fig. 4d).

The cytokine responses to gC-1 and gE-1 did not vary between asymptomatic and symptomatic HSV-2-infected individuals (not shown). Furthermore, the levels of IL4 and IL10 were low overall (not shown). The levels of IL6 ranged from 4 to 10,000 pg ml\(^{-1}\), but there were no differences between the asymptomatic and symptomatic groups for either of the antigens tested (not shown).

The Th1 cytokine responses varied within the asymptomatic group of HSV-2-infected individuals (not shown). Eight of the 21 asymptomatic individuals examined produced high levels of Th1 cytokines, whereas the other 13 produced levels that were similar to those obtained in the symptomatic group. We have no explanation for this heterogeneity. There was no correlation with HSV-1 co-infection or with their antibody responses to sgG-2 or mgG-2. However, these eight individuals had a statistically higher proliferative response to all the HSV antigens tested (compared with the other 13 asymptomatic individuals), but not to PHA. Furthermore, they also produced higher levels of gG-2-specific IL10.

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The cytokine responses to gC-1 and gE-1 did not vary between asymptomatic and symptomatic HSV-2-infected individuals (not shown). Furthermore, the levels of IL4 and IL10 were low overall (not shown). The levels of IL6 ranged from 4 to 10,000 pg ml\(^{-1}\), but there were no differences between the asymptomatic and symptomatic groups for either of the antigens tested (not shown).

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or HSV-1 disease status (Table 1). Finally, we determined whether there was any correlation between the humoral and cell-mediated immune responses to gG-2. The T-cell proliferative responses to sgG-2 or mgG-2 did not correlate with either the anti-sgG-2 or the anti-mgG-2 IgG titres (data not shown).

**DISCUSSION**

In this study, we have shown that the CD4⁺ T-cell response to both HSV-2 sgG-2 and mgG-2 is type specific for HSV-2 and can thus, as with the humoral response to this protein, be used both to distinguish between HSV-1- and HSV-2-infected individuals and to analyse the HSV-2-specific T-cell response in HSV-1/HSV-2 dual-infected individuals. Furthermore, we found that the Th1 cytokine response obtained against these two proteins differed significantly in relation to disease status. The Th1 cytokine responses to sgG-2 and mgG-2 were significantly lower in HSV-1-negative patients with recurrent symptomatic HSV-2 disease compared with asymptomatic HSV-2 carriers. In contrast, the T-cell responses to the HSV-1 envelope glycoproteins gC-1 and gE-1 were not type specific. However, the T-cell proliferative responses to gC-1 and gE-1 were significantly stronger in symptomatic HSV-2 patients carrying a concomitant HSV-1 infection than in HSV-1-negative symptomatic patients or in those carrying an asymptomatic HSV-2 infection.

This is the first report to show T-cell reactivity to sgG-2. We have recently shown that there is a type-specific antibody response to this protein in HSV-2-infected patients (Görander et al., 2003). The cellular response to sgG-2 was also type specific, i.e. T cells from HSV-2-infected, but not from HSV-1-infected, individuals proliferated in response to sgG-2. That the response to sgG-2 is HSV-2 type specific is not surprising as the major part of the protein lacks a corresponding homologue in HSV-1. We also showed that mgG-2 induced an HSV-2 type-specific T-cell response. Neither HSV-1-infected nor HSV-seronegative controls responded to mgG-2. The amino-terminal half of mgG-2, which is heavily O-glycosylated, is also a unique sequence for HSV-2. In contrast, the carboxy-terminal part of mgG-2 shares an overall 50% residue identity with gG-1. Interestingly, the human anti-mgG-2 antibody response was mainly localized to the homologous region, and mapping of linear epitopes within gG-1 and mgG-2 using the pepscan technique has shown that the type-specific reactivity was maintained despite the fact that the antibodies recognized a region with high similarity (9 of 14 identical residues) between the gG proteins (Liljeqvist et al., 1998; Tunbäck et al., 2000). Despite the existence of several short regions with high amino acid similarity between gG-1 and the carboxy-terminal part of mgG-2, the T-cell response was type specific, suggesting that a few type-specific gG-1 or mgG-2 residues are sufficient to maintain the type-specific T-cell recognition. An alternative explanation, considering that the localization of the T-cell epitopes on mgG-2 is unknown, is that the T-cell epitopes are localized to the amino-terminal part of mgG-2.

Our data indicated that recurrent symptomatic disease is
associated with a low Th1 cytokine response to HSV-2. Even though PBMC from asymptomatic and symptomatic individuals were comparable with respect to sgG-2- and mgG-2-specific proliferative capacity, the PBMC from symptomatic patients produced significantly lower levels of IFN-\(\gamma\), TNF-\(\alpha\) and IL2 in response to these proteins. Thus, whereas the Th1 cytokine responses among the asymptomatic carriers increased proportionally with increased proliferation, the sgG-2-specific and mgG-2-specific Th1 cytokine responses remained low among the symptomatic patients. These differences were especially impressive among the HSV-1-negative individuals. These data corroborate and extend previous observations that there is an impairment in the HSV-specific IFN-\(\gamma\) response among patients with symptomatic disease (Burchett \textit{et al}., 1992; Singh \textit{et al}., 2003). IFN-\(\gamma\), IL2 and TNF-\(\alpha\) have all been ascribed important roles in protection against HSV-2 infection. TNF-\(\alpha\), and in particular IFN-\(\gamma\), are instrumental in the activation of macrophages, which play a key role in the early inflammatory response to HSV (Heise & Virgin, 1995). IFN-\(\gamma\) can be induced \textit{in vivo} by administration of recombinant IL2, which protects neonatal mice from a lethal HSV infection (Kohl \textit{et al}., 1989). Similarly, mice lacking IFN-\(\gamma\) are highly susceptible to genital HSV-2 infection and are unable to mount a protective immune response to HSV-2 following vaccination (Harandi \textit{et al}., 2001).

In recent years, it has become increasingly evident that the strength of the immune response is controlled by a small subset of T cells, the CD4\(^+\)CD25\(^+\) regulatory T cells (T-reg). T-reg have an overall suppressive effect on specific T-cell responses (Suri-Payer \textit{et al}., 1998). T-reg are induced during persistent infections and are instrumental in controlling both the immune response and the infection (Belkaid \textit{et al}., 2002). The presence of T-reg also appears to be required to maintain a long-term memory of the infectious agent (Belkaid \textit{et al}., 2002). Experimental studies of HSV infection show that the number of HSV-reactive T cells is negatively correlated with the presence of T-reg (Suvas \textit{et al}., 2003). Thus, T-reg clearly play a role in controlling the strength of the HSV-specific T-cell response. We suggest that symptomatic HSV-2 infection might reflect an imbalance in the HSV-specific T-reg population leading to a specific inhibition of Th1 cytokine development. The factors that influence the development, numbers and strength of T-reg are as yet unknown.

We found that symptomatic individuals carrying a concomitant HSV-1 infection responded significantly more strongly than asymptomatic carriers to gC-1 and gE-1. Thus, the T-cell responses to gC-1 and gE-1 were more pronounced in symptomatic HSV-2-infected patients compared with asymptomatic HSV-2 carriers and were not influenced by the status (clinical or subclinical) of the HSV-1 infection. This is to our knowledge the first report showing a different T-cell proliferative responsiveness to HSV antigens in asymptomatic and symptomatic HSV-2-infected individuals. Similar observations have previously

**Table 1.** Serum antibody responses to sgG-2 and mgG-2 in HSV-2-infected individuals

<table>
<thead>
<tr>
<th>Disease status</th>
<th>HSV-1 status</th>
<th>Anti-sgG-2 Ab titre (median)</th>
<th>Anti-mgG-2 Ab titre (median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic</td>
<td>+</td>
<td>&lt;100–6400 (800)</td>
<td>&lt;100–6400 (800)</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>–</td>
<td>&lt;100–1600 (400)</td>
<td>100–6400 (800)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>+</td>
<td>&lt;100–6400 (800)</td>
<td>&lt;100–12800 (600)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>–</td>
<td>100–1600 (300)</td>
<td>&lt;100–1600 (800)</td>
</tr>
</tbody>
</table>
been reported in HSV-1-infected individuals, where symptomatically HSV-1-infected individuals were found to respond to ICP8 and VP16, whereas those with asymptomatic HSV-1 infection did not (Spatz et al., 2000).

We can only speculate why symptomatic HSV-1/HSV-2-infected individuals have stronger gC-1 and gE-1 responses than asymptomatic HSV-2 carriers, while there is no difference in their proliferative responses to sgG-2 and mgG-2. It is generally accepted that HSV-1 is acquired prior to HSV-2. Our data would imply that a clinically apparent infection with HSV-2 would boost the already existing anti-HSV-1 T-cell response, whereas a subclinical HSV-2 infection would not. Alternatively, the strength of the proliferative T-cell response to the HSV-1 infection will predict the outcome of the HSV-2 infection: the stronger the T-cell response to HSV-1, the higher the risk of developing symptoms when infected by HSV-2.

In summary, we have shown that sgG-2 and mgG-2 are type specific for HSV-2-specific T-cell responses and that asymptomatic and symptomatic HSV-2 infections could be distinguished by their Th1 cytokine profiles in response to sgG-2 and mgG-2. This implies that there is a difference in either the induction or the maintenance of HSV-specific T-cell responses in asymptomatic and symptomatic HSV-2 infection.

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REFERENCES


