Increased Cell-Mediated Immune Responses in Patients with Recurrent Herpes Simplex Virus Type 2 Meningitis

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The clinical picture of herpes simplex virus type 2 (HSV-2) infection includes genital blisters and less frequently meningitis, and some individuals suffer from recurrent episodes of these manifestations. We hypothesized that adaptive and/or innate immune functional deficiencies may be a major contributing factor in susceptibility to recurrent HSV-2 meningitis. Ten patients with recurrent HSV-2 meningitis were studied during clinical remission. For comparison, 10 patients with recurrent genital HSV infections as well as 21 HSV-seropositive and 19 HSV-seronegative healthy blood donors were included. HSV-specific T cell blasting and cytokine secretion were evaluated in whole blood cultures. HSV-2-induced NK cell gamma interferon production, dendritic cell Toll-like receptor (TLR) expression, and TLR agonist-induced alpha interferon secretion were analyzed. Patients with recurrent HSV-2 meningitis had elevated T cell blasting and Th1 and Th2 cytokine production in response to HSV antigens compared to those of patients with recurrent genital infections. A somewhat increased NK cell response, increased dendritic cell expression of TLR3 and -9, and increased TLR-induced alpha interferon responses were also noted. Contrary to our expectation, recurrent HSV-2 meningitis patients have increased HSV-specific adaptive and innate immune responses, raising the possibility of immune-mediated pathology in the development of recurrent HSV2 meningitis.

Benign recurrent aseptic meningitis of unknown cause, called Mollaret’s syndrome, was first described in 1944 (30). The disease is characterized by recurrent periods of neck stiffness, headaches and fever, and the presence of large cells in cerebrospinal fluid (CSF). Since the development of the PCR technique, the causative agent of recurrent meningitis (RM) is generally considered to be herpes simplex virus type 2 (HSV-2) (22, 36, 43), which is readily detectable in CSF. The first episode of HSV-2 meningitis usually develops within 2 weeks following symptomatic or asymptomatic primary genital HSV-2 infection (8, 37). HSV-2 meningitis is more common in women than in men, with a reported incidence of 36% in women and 13% of men among patients with primary genital HSV-2 infection (8). After a first episode of HSV-2 meningitis, the incidence of recurrent meningitis varies between studies but is around 30% (1, 6, 37).

HSV infects submucosal nerve endings in oral or genital tissue and establishes latency in nerve ganglia such as trigeminal ganglion in oral HSV-1 infection and sacral dorsal root ganglion in genital HSV-2 infection. Viral reactivation occurs frequently and is induced by events such as hormonal changes, stress, or other diseases. It results in viral transport along the axon and shedding of virus at mucosal surfaces, in most cases without causing clinical symptoms (27, 47). In HSV-2 meningitis, viral entrance to CSF has assumed to be by seeding from infected ganglia, but a recent report of frequent viremia during primary genital infection suggests the possibility of viral spread to the central nervous system (CNS) from blood (15).

Immune suppression associates to symptomatic viral reaction, and gamma interferon, produced mainly by CD4+ T cells (45) but also by CD8+ T cells (25), and granzymes (19) have been described to be of importance in combating viral production. HSV-specific cytotoxic T cells can be demonstrated in latently infected ganglia (46) as well as near infected nerve endings in mucosa (52). As for specific T cell responses in HSV-2 infections, a number of glycoproteins have been described as immunodominant, including envelope gB, gC, and gD (20, 21, 29), in some cases even in the absence of detectable antibodies in serum (34). Innate immune mechanisms important in preventing primary HSV infection have been identified, genetically and functionally defining naturally occurring immune deficiencies in familial HSV-1 encephalitis. NK cells (31), as well as Toll-like receptors (TLR) and alpha interferon (IFN-α) responses, play vital roles (50). However, there is no information available on HSV-specific adaptive or innate cellular immune responses in patients with recurrent HSV meningitis, and such data are important to better understand the pathogenesis of this disease. Interestingly, Aurelius found that 61 of 64 consecutive cases of primary and recurrent HSV-2 meningitis lacked antibodies to HSV-1 (2), suggesting that a
major risk factor for developing meningitis was the absence of prior HSV-1 infection and, hence, absence of a prior immune response to HSV.

As literature on cell-mediated immune responses in patients with recurrent HSV-2 meningitis is lacking, we set forth to characterize immune responses in this disease with the aim to detect immune defects or deviations in patients with recurrent HSV-2 meningitis that may help to explain why some individuals get meningitis as a complication to viral reactivation. We investigated immune responses in 10 patients with recurrent and severe manifestations of HSV-2 meningitis, as well as in 10 patients with recurrent genital HSV-2 infection (Figure 1A) and in 21 HSV-seropositive and 19 HSV-seronegative healthy controls. Our data show increased expression of Toll-like receptors, increased IFN-α responses, and increased specific T cell responses in patients with HSV-2 meningitis. Thus, patients with recurrent meningitis have elevated rather than decreased immune responses. One interpretation of our data is that a strong antiviral immune response, such as high levels of cytokines, can contribute to pathology and viral spread to the meninges.

### Materials and Methods

#### Subjects

Ten patients with recurrent HSV meningitis were included in the study (Table 1). Exclusion criteria were ongoing symptomatic herpes simplex infection, acute febrile illness, other chronic diseases, immunosuppressive treatment, or vaccination within a month prior to inclusion. All patients included were asymptomatic at the time of inclusion.

#### Herpes simplex virus serology

Anti-HSV antibodies were detected using the HerpeSelect enzyme-linked immunosorbent assay (ELISA) (Focus Technologies, Cypress, CA) for qualitative detection of type-specific IgG antibodies against recombinant HSV-1 gG-1 or HSV-2 gG-2 according to the manufacturer's instructions.

#### Enumeration of blood cells and FACS staining

Cells were stained for CD3, CD4, CD8, CD16/56, TCR-α/β, TCR-γ/δ, HLA-DR, CD14, CD16, CD19, CD20, CD123, CD11c, TCR, and CD45 and analyzed by flow cytometry (FACS) using TrueCount tubes (BD Biosciences, San Diego, CA), allowing determination of absolute cell counts in blood (CellQuest Pro and MultiSET software and all antibodies from BD Biosciences).

### Table 1. Clinical description of 10 patients with recurrent HSV-2 meningitis

| Gender | Age (yr) | HSV antibodies(+)a | Time (yr) since first meningitis | No. of recurrences | Time (yr) since last meningitis | Laboratory diagnosed | Clinically suspected |
|--------|----------|---------------------|---------------------------------|--------------------|-------------------------------|----------------------|--------------------|
| Female | 49       | HSV-2               | 6                               | 4                  | 0                             | 6 mo                 |                    |
| Female | 29       | HSV-2               | 5                               | 3                  | 3                             | 6 mo                 |                    |
| Female | 41       | HSV-2               | 3                               | 4                  | 8                             | mo                   |                    |
| Female | 29       | HSV-2               | 5                               | 15                 | 15                            | 3 mo                 |                    |
| Female | 51       | HSV-2               | 33                              | 7                  | 1                             | 1 mo                 |                    |
| Female | 39       | HSV-2               | 10                              | 4                  | 6                             | 5 mo                 |                    |
| Female | 62       | HSV-2               | 33                              | 5                  | 20                            | 6 mo                 |                    |
| Male   | 38       | HSV-2               | 15                              | 6                  | 58                            | 2 wk                 |                    |
| Female | 49       | HSV-2               | 26                              | 5                  | 2                             | 4 mo                 |                    |
| Female | 51       | HSV-1,-2            | 13                              | 7                  | 18                            | 10 mo                |                    |

a The presence of HSV-1 or -2 was confirmed by PCR or culture of cerebrospinal fluid in the first three to seven episodes of each patient, as indicated. All patients included were asymptomatic at the time of inclusion.

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**TABLE 2. Clinical description of 10 patients with recurrent HSV genitalis**

| Gender | Age (yr) | HSV antibodies(+)a | Time (yr) since first episode | No. of recurrences |
|--------|----------|---------------------|------------------------------|--------------------|
| Female | 33       | HSV-2               | 4                             | 4                  |
| Male   | 36       | HSV-2               | 6                             | 4                  |
| Male   | 54       | HSV-2               | 2                             | 18                 |
| Male   | 44       | HSV-1,-2            | 16                            | 8                  |
| Female | 33       | HSV-1,-2            | 6                             | 2                  |
| Male   | 28       | HSV-1,-2            | 8                             | 3                  |
| Male   | 33       | HSV-1,-2            | 8                             | 3                  |
| Female | 34       | HSV-2               | 12                            | 12                 |
| Male   | 40       | HSV-2               | 17                            | 4                  |

a The presence of HSV-1 or -2 was confirmed by PCR or culture of samples taken from the blisters. All patients included were asymptomatic at the time of inclusion.
RESULTS

HSV-specific adaptive immune responses are elevated in patients with recurrent meningitis. We analyzed HSV-specific immune responses to two different antigen preparations in patients with RM. One preparation consisted of HSV-1 nucleocapsid, and the other was a whole-cell lysate of HSV-2-infected cells, which includes nucleocapsid but also viral envelope antigens. The two antigen preparations were shown to elicit responses in HSV-1- as well as HSV-2-infected persons. Seronegative specimens did not respond (Fig. 1, Table 3). As controls, patients with recurrent genital HSV infection were included. Patients with recurrent HSV infections (RM or RG) had a highly significant increased production of Th1 cytokines such as IL-12, IL-10, and TNF-α compared to those of seropositive blood donors (Table 3). Interestingly, RM patients responded to both antigen preparations with higher levels of IL-4 and IL-13 than those of seropositive blood donors and RG patients (Table 3). In addition, the T cell blast response as well as the production of several cytokines upon stimulation with the whole-cell lysate was higher for the RM group than for the RG group. These data show that patients with recurrent HSV-2 meningitis develop stronger rather than weaker responses to HSV antigens than seropositive patients without a documented history of meningitis or patients with recurrent genital HSV infection.

Increased innate immune responses in patients with meningitis. RM and RG patients had comparable numbers of NK cells (cells/μl blood: RG, 139 [range, 6 to 437]; RM, 218 [110 to 312]) in peripheral blood. Freshly analyzed NK cells did not show any phenotypical differences between patient groups as defined by the expression of NK2D, DNAM-1, Nkp30, Nkp44, Nkp46, and CD69, and NK cell cytotoxicity against the classical target cell line K562 was similar in all groups (data not shown). However, NK cells from RM patients, compared to those from RG patients, presented a somewhat increased IFN-γ production when cultured in the presence of HSV-2 live virus, although this difference was not statistically significant from the upper and lower values of the box; outliers and extremes are not shown in the graph, and the median values are indicated within the boxes.

![FIG. 1. Specific response to HSV-2 whole-cell lysate stimulation.](image-url)

Median and 25th to 75th percentiles of the numbers of CD4+/H11001 lymphoblasts generated per μl of whole blood from HSV-seropositive (sero+) and -seronegative (sero–) donors cultured 7 days in the presence of HSV-2-infected whole-cell lysates. The Mann-Whitney U test was used for statistical analysis. *,* P < 0.05.

| Cytokine | HSV– (D) | HSV+ (D) | HSV+ (RM) | HSV+ (RG) |
|----------|----------|----------|-----------|-----------|
| IL-2     | 8.4 (3.8–10.4) | 12.1 (9.3–16.1) | 11.1 (9.3–13.8) | 10.4 (8.7–12.1) |
| IL-4     | 0.0 (0.0–0.0) | 0.6 (0.1–1.3) | 0.5 (0.1–0.6) | 1.3 (0.9–2.1) |
| IL-6     | 2.4 (0.0–3.5) | 121 (63–463) | 421 (167–1,296) | 252 (215–377) |
| IL-9     | 0 (0–0) | 2.3 (0.0–3.1) | 2.7 (0.0–3.6) | 5.2 (3.9–7.5) |
| IFN-α    | 0 (0–0) | 14 (2.8–45) | 96 (59–213) | 74 (53–151) |
| IFN-β    | 0 (0–0) | 14 (2.8–45) | 96 (59–213) | 74 (53–151) |
| IL-12    | 0 (0–0) | 0 (0–0) | 4.9 (1.1–5.5) | 5.1 (4.2–5.6) |
| IL-13    | 0 (0–0) | 0 (0–0) | 5.3 (1.1–6.6) | 5.5 (4.7–6.7) |
| IL-17    | 0 (0–0) | 12 (2.4–20) | 40 (32–54) | 112 (64–149) |
| GM-CSF   | 0.0 (0–1.3) | 11 (3.1–62) | 11 (4–64) | 42 (23–66) |
| IL-10    | 0 (0–0) | 1.9 (0.0–4.5) | 7.3 (5.7–16) | 16 (10–19) |
| TNF-α    | 0 (0–0) | 301 (97–1740) | 226 (193–2,987) | 221 (81–654) |
| IL-8     | 2.3 (0.0–6.5) | 22 (4.0–10.5) | 22 (4.0–10.5) | 22 (4.0–10.5) |
| IL-15    | 0 (0–0) | 0.0 (0–0) | 0 (0–0) | 0 (0–0) |
| IL-18    | 0 (0–0) | 8.4 (3.8–10.4) | 12.1 (9.3–16.1) | 11.1 (9.3–13.8) |
| IL-21    | 0 (0–0) | 0.0 (0–0) | 0 (0–0) | 0 (0–0) |

**TABLE 3. HSV-specific cell-mediated immune responses**
Since virally induced NK cell production of IFN-γ is probably a consequence of accessory cell activation, we also characterized the dendritic cell (DC) phenotype of RM patients. The numbers of plasmacytoid DCs (pDC) and myeloid DCs (mDC) did not differ between RM and RG patients (cells/μl blood for RM: pDC, 8 [3 to 20], and mDC, 11 [5 to 22]; for RG: pDC, 6 [1 to 15], and mDC, 14 [9 to 23]). To further characterize differences in DC function, we examined by flow cytometry the expression of intracellular TLR3, TLR7, and TLR9. Unfortunately, we did not have access to blood from RG patients in this characterization, and therefore a comparison of RM was done only with healthy HSV-2-seropositive controls. We found an increased expression of TLR3 levels in RM patients, higher IFN-γ production from PBMCs cultured in the presence of different TLR agonists [lipopolysaccharide (LPS), CpG ODN2216, HSV-2]. In accordance with elevated TLR3 levels in RM patients, higher IFN-α levels could be detected in RM samples than in those from healthy seropositive controls when stimulated with poly(I:C) (Fig. 2C). There were no significant differences in responsiveness to the other TLR agonists used (data not shown).

**DISCUSSION**

It is not known why some patients with HSV-2 infection develop recurrent meningitis. Data on innate and adaptive immune responses in patients affected are scarce, and ours is the first study of HSV-specific immune responses in patients with recurrent HSV-2 meningitis. We show for the first time that these patients have increased systemic immune responses compared to those of patients with recurrent genital HSV infections and to those of healthy seropositive individuals. The difference includes both innate and adaptive immune responses. We describe higher antigen reactivity, measured as CD4^+ T cell blasting and cytokine production. In particular, the HSV lysate induced elevated Th1 and Th2 cytokine responses. In addition, a higher level of expression of TLR3 and TLR9, along with increased IFN-α production in response to a TLR3 agonist, was detected in meningitis patients than in seropositive healthy controls.

Patients with recurrent HSV-2 meningitis as well as patients with recurrent genital HSV-2 infection displayed stronger HSV-specific cell-mediated immune responses than healthy seropositive subjects, in particular regarding production of cytokines IL-12, TNF-α, and IL-10. One likely explanation is that frequent symptomatic recurrences provide repeated and high-dose exposure of HSV to the adaptive immune system, inducing an increased response, including cytokines such as IL-12 and TNF-α associated with Th1 profiles. This proinflammatory response may be one explanation for the more severe clinical symptoms seen in our two patient groups than in the seropositive donor group. Although seropositive healthy blood donors have frequent periods of viral secretion (27) and we expect that a minority of the healthy controls included have symptomatic infections, the total and cumulative viral dose may be lower in these donors than in the patient groups selected for frequent symptomatic reactivations.

Another possible cause of a higher T cell response in patients with meningitis than in healthy seropositive donors is a higher inherent innate function in the patients. To investigate this possibility, we analyzed TLR expression and responses in blood myeloid cells, since the production and function of IFN-γ and functional TLRs (7) have been shown to play essential roles in protection against primary HSV-1 encephalitis. We show that in addition to elevated T cell responses and cytokine production, expression of TLR3 and -9 is increased in DCs from meningitis patients sampled during clinical remission compared to that in DCs from healthy HSV-2-seropositive donors. To examine whether this increased expression of TLR3 results were seen for mDC but not for monocytes (data not shown).
donors. There are several reports showing changes in TLR expression levels during infectious disease and inflammation (24, 26, 33). Several animal studies document an importance of TLRs in controlling antiviral responses against, for example, murine cytomegalovirus (41, 42). Moreover, TLR3 and related molecules (51) have been shown to be indispensable in the control of herpes simplex encephalitis. The increased innate responses may indicate that patients developing recurrent meningitis are genetically different from those who do not develop recurrent meningitis, and we cannot exclude that TLR3 deficiencies are present in our group of patients, as such defects might be underestimated in our experimental setup. In fact, in a study characterizing genetic defects in IFN-α production, the TLR3 defect could be detected only by using fibroblasts and not peripheral blood cells (7). Another possibility would be that these differences of innate immunity could be the result of the persisting and recurrent HSV-2 meningitis, and to analyze this, one would need to characterize innate immunity in persons before they acquire HSV-2 meningitis. Additionally, NK cell function was somewhat elevated in meningitis patients compared to that in patients with genital recurrences, and it is possible that this reflects the elevated TLR reactivity of meningitis patients’ DCs. Alternatively, it may be a reflection of a recently described long-lasting priming of NK cell function by viral activation (38).

We have found no support in the literature that immune mechanisms affected by, for example, medical immune suppression or HIV infection are involved in protection against HSV meningitis. However, the pathology of genital HSV-2 is clearly controlled by such mechanisms, since genital symptoms are increased on immune suppression (35). We speculate that the type of immune response elicited upon viral reactivation affects clinical outcome, with a strong response inducing pronounced immunopathological symptoms. An increased local IL-13 production in the dorsal ganglia during viral reactivation may perhaps result in tissue remodeling by IL-13-induced disruption of tight junctions (12). This could allow virus and T cells to pass through the blood-brain barrier. Clearly, oligodendroglial T cells are present in infected ganglia (14, 46) even during latency (44), and virus may enter either via neuron-neuron interactions or from circulation during viremia (15). A higher incidence in women may, in this view of immune-mediated pathology, reflect a generally higher immune reactivity in females (8, 11). Accordingly, healthy women have been shown to respond more efficiently upon stimulation through TLR7 (28), with a subsequent higher T cell response, than men. An example of a viral infection in the CNS with proposed immune-mediated pathology is the lymphocytic choriomeningitis virus (LCMV). Here, cytotoxic T cells promote destruction of the blood-brain barrier through the release of cytokines and chemokines that facilitates recruitment of myelomonocytic cells with consequent vascular leakage and disease progression (18). It is thus possible that such immune-mediated pathology could be more prominent in women than in men.

Johnston et al. recently demonstrated that HSV viremia is common during primary genital infection (15) and thus virus may spread to the central nervous system from blood during primary infection and establish latency in extrasacral neurons from which reactivated HSV repeatedly could be seeded to the subarachnoidal space and cause recurrent meningitis. Furthermore, development of meningitis when contracting HSV-2 is very rare in persons with already established latent HSV-1 infection (3) (Table 1), indicating that adaptive immunity to type-common HSV antigens can provide protection against development of HSV meningitis disease. The prevalence of HSV-1 and HSV-2 varies with age and geographical region. In Europe, HSV-1 is generally acquired during childhood, reaching a prevalence of up to 80 to 90% in adults, whereas HSV-2 is acquired mostly in adolescence and early adulthood, with a prevalence of up to 25% (32). A declining prevalence of HSV-1 has been recorded in adolescents in the United States during the last decades (49). This changing epidemiology of HSV infections suggests an increasing incidence of primary HSV-2 infections in HSV-1-negative persons, which has been reported to increase the likelihood of symptomatic genital infection (2, 23, 48) as well as meningitis (3). The cohort of recurrent meningitis patients included in our study confirms that HSV-2 meningitis is more common in patients without prior HSV-1 infection (Table 1). It could be that the absence of HSV antibodies in a subject newly exposed to HSV-2 can result in a higher viral titer and a stronger immune response that promotes viral spread to the CNS, thus contributing to meningitis. Based on this, we expect an increasing prevalence of HSV-2 meningitis in the future, making a better understanding of the pathogenesis of HSV-2 meningitis even more important.

In conclusion, we have characterized HSV-specific immune responses in patients with recurrent meningitis, in comparison to patients with recurrent genital infections, and found a higher innate function and HSV-specific T cell reactivity. The incidence of HSV-2 meningitis has probably been underestimated (2, 10, 17) and may increase in the future. Although local immune responses in CNS may be different to responses in peripheral blood, our data provide new information on the agent-host interaction in recurrent HSV-2 meningitis and may be valuable for designing future strategies for antiviral and/or immunological treatment of patients with this disease.

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We report no conflicts of interest.

REFERENCES

1. Afonso, N., et al. 2007. Appropriate use of polymerase chain reaction for detection of herpes simplex virus 2 in cerebrospinal fluid of patients at an inner-city hospital. Diagn. Microbiol. Infect. Dis. 57:309–313.
2. Aurelius, E. 2006. Neurological disease in herpes simplex virus type 2 infection, p. 317–336. In M. Studahl, P. Cinque, and T. Bergström (ed.), Herpes simplex viruses. Taylor and Francis Group, New York, NY.
3. Aurelius, E., M. Forsgren, E. Gille, and B. Skoldenberg. 2002. Neurologic morbidity after herpes simplex virus type 2 meningitis: a retrospective study of 40 patients. Scand. J. Infect. Dis. 34:278–283.
4. Aurelius, E., B. Johansson, B. Skoldenberg, and M. Forsgren. 1993. Encephalitis in immunocompetent patients due to herpes simplex virus type 1 or 2 as determined by type-specific polymerase chain reaction and antibody assays of cerebrospinal fluid. J. Med. Virol. 39:179–186.
5. Aurelius, E., B. Johansson, B. Skoldenberg, A. Stolz, and M. Forsgren. 1991. Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. Lancet 337:189–192.
