Quantitative analysis of herpes simplex virus type 1-specific memory B cells generated by different routes of infection

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Abstract

We compared the herpes simplex virus type 1 (HSV)-specific memory B cell (MBC) populations generated by footpad and intranasal infection in mice. Both routes of infection generated transient antibody-secreting cell responses in the draining lymph nodes and spleen, and sustained circulating IgG. HSV-specific IgG MBCs, analyzed by limiting dilution assay approximately 8 weeks after infection, were distributed in a range of lymph nodes and in the spleen and Peyer’s patches. Overall, the route of infection had little effect on the MBC frequency in each anatomical location. Interestingly, after both routes of infection there was a trend towards preferential MBC accumulation in the mediastinal lymph node. Intravaginal challenge of mice primed by footpad or intranasal infection generated similar secondary IgG responses. Our findings indicate that the widespread dispersion of MBCs to lymphoid tissues throughout the body is largely independent of the route of infection, but may be influenced by tissue-specific factors.

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Introduction

The B cell response to infection and vaccination generates long-lived plasma cells (LLPCs) and memory B cells (MBCs), the two cellular components of B cell memory (McHeyzer-Williams and McHeyzer-Williams, 2005). LLPCs are typically recognized as bone marrow (BM)-resident cells that are primarily responsible for maintaining high circulating antibody (Ab) levels (Hyland et al., 1994; Sangster et al., 1995; Slifka et al., 1995, 1998). Plasma cells may also traffic to mucosal sites and establish long-lasting populations that produce locally protective Ab (Jones and Ada, 1987; Liang et al., 2001). MBCs are also long-lived (Schittek and Rajewsky, 1990) but, unlike plasma cells, they are quiescent, non-Ab-secreting cells that circulate and distribute to organized lymphoid tissues throughout the body (Bachmann et al., 1994; Blink et al., 2005; Crotty et al., 2003). Upon exposure to “recall” antigen (Ag), MBCs divide and differentiate into plasma cells and mediate the rapid, vigorous, and high-affinity secondary Ab response. In this way, the MBC response supplements pre-existing Ab and contributes substantially to Ab-mediated protection. Non-specific activation of MBCs by a variety of signals may also contribute to the maintenance of long-lasting plasma cell populations (Bernasconi et al., 2002).

Quantitative aspects of MBC populations generated by viral infection and vaccination have received little attention. This is important information, since the number and location of MBCs may be significant determinants of protective immunity. Notably, Slifka and Ahmed (1996) applied a limiting dilution assay (LDA) to determine virus-specific MBC frequencies after lymphocytic choriomeningitis virus (LCMV) infection in a mouse model. LCMV-specific MBCs circulated in the blood and established frequencies of approximately 1 in 10^4 lymphocytes in lymph nodes and spleen (Slifka et al., 1998). However, similar analyses of other viral systems have not been undertaken. The extent of MBC dispersion is not well established, and the effect of virus type and route of infection has not been investigated.
Herpes simplex virus type 1 (HSV) is an important human pathogen that typically replicates in epithelial cells prior to establishing latency in sensory neurons (Khanna et al., 2004). The virus can produce both cutaneous and mucosal infections, since the epithelial cells targeted include epidermal cells of the skin and cells of mucosal surfaces. These different forms of infection can be modeled in the mouse by administering the virus via different routes (Biswas and Rouse, 2005; Pereira et al., 2001; Reading et al., 2006; Simmons et al., 1997). We have recently developed an LDA for the determination of HSV-specific MBC frequencies (Li et al., 2006). In the current study, we apply this assay in a quantitative analysis of the HSV-specific MBC pools generated in the mouse model by footpad infection or by infection of the respiratory tract. Our findings indicate that the dispersion and response of HSV-specific MBCs is largely unaffected by the route of immunization. Nevertheless, lymphoid tissue preferences in MBC localization were identified.

Results

HSV-specific antibody response to cutaneous and mucosal infection

Initial experiments determined the kinetics of HSV-specific Ab production following two forms of infection: cutaneous infection (footpad inoculation) and infection of the respiratory tract [intranasal (i.n.) inoculation]. The ELISPOT assay was used to enumerate HSV-specific Ab-secreting cells (ASCs) in different tissues. Footpad infection generated responses in the draining popliteal lymph node (PLN) and the spleen characterized by an early peak of IgM ASCs, followed by increased numbers of cells producing IgG isotypes, with IgG2b and IgG2c predominating (Figs. 1A and B). By day 30, responses in the PLN and spleen had substantially waned. However, at this time point a population of IgG ASCs was established in the BM (Fig. 1C). IgA ASCs were essentially absent from the PLN response, and present at only very low frequencies in the spleen and later in the bone marrow. The pattern of responses to i.n. infection resembled that following footpad infection, but was delayed by 2–4 days in the draining lymph nodes and spleen (Fig. 2). Notably, i.n. infection generated an IgA response. This was most apparent when nasal-associated lymphoid tissue (NALT), a well-established IgA inductive site (Liang et al., 2001), and cervical lymph nodes (CLN) were sampled on day 8 (Fig. 2E), indicating that transient IgA induction occurs early in the response.

Virus-specific IgG in serum plateaued at similar levels after footpad and i.n. infection (Fig. 3A). However, the initial increase was more rapid after footpad infection, correlating with the kinetics of the ASC response. The low levels of virus-specific IgA in serum also correlated with IgA ASC formation. Only i.n. infection resulted in virus-specific IgA in vaginal secretions (Fig. 3B), emphasizing the importance of mucosal antigen administration in the induction of IgA responses.

Fig. 1. The HSV-specific ASC response to footpad infection. The kinetics of virus-specific ASC responses in the PLN (A), spleen (B), and bone marrow (C) were determined for B6 mice infected in the footpad with HSV. Cell suspensions were prepared from individual mice and the ELISPOT assay was used to enumerate HSV-specific ASCs. Results are expressed as the number of ASC/1×10^5 or 5×10^5 nucleated cells. The mean±SE is shown for 3 mice.

HSV-specific MBCs generated by cutaneous and mucosal infection

A newly developed LDA (Li et al., 2006) was used to determine HSV-specific IgG MBC frequencies in a range of lymphoid tissues 45–65 days after footpad and i.n. infection. At this time, the ASC response had waned and a stable distribution of MBCs would be expected (Bachmann et al., 1994; Slifka et al., 1998). Following both routes of infection, HSV-specific MBCs were detected in all lymphoid tissues examined (Fig. 4), consistent with the concept of a widespread dispersion of these cells. MBC frequencies in the PLN were generally higher following footpad compared with i.n. infection, perhaps reflecting the central role of the PLN in responding to Ag deposited in the footpad. Otherwise, however, the route of infection had little effect on the MBC frequencies established in each anatomical location, suggesting an additional influence of local tissue factors on MBC distribution patterns. An interesting trend after both routes of infection was the preferential localization of HSV-specific MBCs in the mediastinal lymph node (MLN). The presence of MBCs in Peyer’s patches (PP) after footpad infection demonstrates that mucosal infection is not a prerequisite for IgG MBC migration to mucosa-associated lymphoid tissues.
To extend our analysis of the MBC pools generated by footpad and i.n. HSV infection, we used intravaginal HSV challenge to evaluate MBC activation in vivo. Mice were held for 50 days after footpad or i.n. HSV infection, and then challenged intravaginally with the virulent McKrae strain of HSV. The ASC response was determined 5 days after challenge in the iliac lymph nodes (IliLN), the primary lymph nodes draining the vaginal tract (King et al., 1998). Naïve mice infected intravaginally had few HSV-specific ASCs in the IliLN on day 5, indicating that the response in the immune mice reflected MBC activation (Fig. 5B). The HSV-specific IgG recall response was not significantly different after footpad or i.n. priming. This result is consistent with the similar IgG MBC frequencies in the IliLN following both routes of priming. The IgG1 component of the recall response was significantly stronger after footpad compared with i.n. priming, raising the possibility of differences in the isotype expression profile of responding IgG MBCs. Interestingly, i.n. priming did not enhance the IgA component in the recall response to intravaginal challenge. Overall, the switched isotype profiles of the primary and recall responses in the IliLN to intravaginal infection remained largely similar (Figs. 5A and B), suggesting a strong element of local regulation of the quality of MBC responses.

Discussion

The current study was undertaken to add to the very limited quantitative information available on the dispersed MBC populations generated by viral infections. We used a mouse model of HSV infection to compare the MBC pools generated by primary infection of the skin (footpad inoculation) and the respiratory tract (i.n. inoculation). The virus replicates well in both of these locations and is cleared with approximately the same kinetics (Jones et al., 2000; Reading et al., 2006). Our analysis demonstrated that the primary infections elicited robust HSV-specific B cell responses in the draining lymph nodes and spleen, and resulted in similar virus-specific bone marrow plasma cell numbers and circulating IgG titers (Figs 1, 2, and 3A). Ag deposition at mucosal surfaces favors IgA formation, and it was therefore not surprising that the early response to i.n. infection included a small IgA component that was absent from the response to footpad infection. Although small, this IgA response was associated with the appearance of HSV-specific IgA in serum and vaginal secretions (Fig. 3). Surprisingly, IgA induction in the respiratory tract was most apparent in the NALT, but was essentially absent from the CLN and MLN, the major sites of IgA production following infection.

MBC response to intravaginal HSV challenge

To extend our analysis of the MBC pools generated by footpad and i.n. HSV infection, we used intravaginal HSV challenge to evaluate MBC activation in vivo. Mice were held for 50 days after footpad or i.n. HSV infection, and then challenged intravaginally with the virulent McKrae strain of HSV. The ASC response was determined 5 days after challenge in the iliac lymph nodes (IliLN), the primary lymph nodes draining the vaginal tract (King et al., 1998). Naïve mice infected intravaginally had few HSV-specific ASCs in the IliLN on day 5, indicating that the response in the immune mice reflected MBC activation (Fig. 5B). The HSV-specific IgG recall response was not significantly different after footpad or i.n. priming. This result is consistent with the similar IgG MBC frequencies in the IliLN following both routes of priming. The IgG1 component of the recall response was significantly stronger after footpad compared with i.n. priming, raising the possibility of differences in the isotype expression profile of responding IgG MBCs. Interestingly, i.n. priming did not enhance the IgA component in the recall response to intravaginal challenge. Overall, the switched isotype profiles of the primary and recall responses in the IliLN to intravaginal infection remained largely similar (Figs. 5A and B), suggesting a strong element of local regulation of the quality of MBC responses.

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of the respiratory tract with non-herpesviruses (Sangster et al., 1997, 2003). HSV resembles murine gammaherpesvirus 68 in the induction of minimal IgA responses in the CLN and MLN following respiratory tract infection (Sangster et al., 2000), raising the possibility that herpesviruses in general may selectively down-regulate IgA responses in some locations. Interestingly, HSV-specific IgA ASCs were present in the BM of footpad-infected mice on day 30, and these cells were presumably responsible for the virus-specific serum IgA that was detected only later in the response. Stochastic factors may play a role in the isotype expression profile of the bone marrow plasma cell population, which does not necessarily reflect that directed by local factors in responding lymph nodes (Hyland et al., 1994).

Previous studies indicate that MBCs migrate via the blood from their sites of formation and, by approximately 3 weeks after immunization, have established frequencies in distant lymphoid tissues that remain stable for many months (Bachmann et al., 1994; Blink et al., 2005; Slifka et al., 1998). Thus, our analysis of HSV-specific MBC populations generated by footpad and i.n. infection was performed when full dispersion and stable frequencies of these cells would be expected (Fig. 4). Our findings are consistent with the concept of widespread dispersion of IgG MBCs, and indicate that the extent of dispersion is independent of the form of Ag exposure. Interestingly, IgG MBCs generated by footpad infection migrated to the mucosa-associated PP, suggesting that non-mucosal vaccination strategies generate MBCs that are well placed to respond to mucosal challenges. Our findings also indicate that the dispersion of MBCs to lymphoid tissues is not equal, and may be influenced by local tissue factors. One factor may be whether the lymphoid tissue participated in the response to infection. For a period of time after infection, MBC frequencies are highest at sites of formation in responding lymphoid tissues. This situation gradually changes as responses wane and MBCs disperse, but the rate of change is likely to be dependent on the experimental system (Bachmann et al., 1994; Baine and Thorbecke, 1982). Thus, in our studies, the site of Ag deposition may have contributed to the trend towards higher
MBC frequencies in the PLN and MLN following footpad and i.n. infection, respectively. Our studies also suggest that other tissue-specific factors may play a role. In particular, this possibility is raised by the trend towards higher MBC frequencies in the MLN compared with some other sites after footpad infection, even though the MLN does not drain the footpad. MBC trafficking patterns and the molecules that regulate this movement are not well defined, and may differ for different MBC subsets (Roy et al., 2002). Since MBCs home to all lymph nodes, even in the absence of ongoing inflammation, one trafficking pattern is likely to be the same as that used by naïve B cells and involve the L-selectin-mediated movement of cells from blood to lymph nodes across high endothelial venules (Kim, 2004). The trend towards higher MBC frequencies in the MLN, regardless of the route of infection, may reflect a tendency for MBCs to also leave the circulation in the lung and enter the MLN via draining lymphatics. This trafficking pathway has been established for CD8+ memory T cells (Zammit et al., 2006).

To further evaluate the distribution of MBCs generated by different routes of HSV infection, immune mice were challenged intravaginally and the secondary B cell response determined in the draining IliLN (Fig. 5). This strategy was used because the route of Ag administration that induces optimal protection against genital HSV infection has been a focus of recent investigations. A vigorous secondary B cell response is likely to contribute significantly to vaginal protection (Morrison et al., 2001; Parr and Parr, 1997), and an important question was whether this is influenced by the route of priming. We found that priming via the footpad or i.n. had little effect on the magnitude of the HSV-specific IgG response after intravaginal challenge. This result is consistent with the similar IgG MBC frequencies in the IliLN after footpad or i.n. infection, although circulating MBCs entering the responding IliLN after challenge may also have contributed to the magnitude of the secondary response. Overall, the impression is that the characteristics of MBC trafficking ensure optimal secondary IgG responses, regardless of the route of priming.

Interestingly, the secondary response to intravaginal challenge included little IgA production, even after i.n. priming. Although we did not determine IgA MBC frequencies in this study, it is likely that IgA MBCs would have been generated in parallel with the IgA response to i.n. infection. The primary HSV-specific response to intravaginal infection typically includes only a small IgA component (Milligan and Bernstein, 1995), and it may be that local tissue factors direct the isotype expression profile of not only the primary response (Sangster et al., 1997), but also that of the memory response. Alternatively, IgA MBCs may distribute differently from IgG MBCs (Moser and Offit, 2001) and may not localize in the IliLN. This important issue has not been resolved.

When the cellularity of the tissues sampled in our study is considered, the spleen contained the vast majority of HSV-specific MBCs, regardless of the route of infection. The count per spleen of approximately 4000–5000 is similar to the number of specific MBCs in this location determined by others following infection with influenza (Li et al., 2006) and LCMV (Slioka et al., 1998). Taken together, these findings suggest a strong similarity in the total number of specific MBCs generated by diverse viruses with different primary sites of replication. Perhaps notably, all of the viral infections studied elicited strong primary B cell responses, and this may be the critical determinant of the size of the MBC pool and the level of protection conferred. It will be of interest to relate the MBC frequencies generated by viral infections to those resulting from various vaccination strategies.

Materials and methods

Mice

C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed under specific pathogen-free conditions until infection, and thereafter in BSL2 containment. Female mice were used in all studies and were first infected at 8–10 weeks of age. The Animal Care and Use Committee of the University of Tennessee approved all animal procedures.

Viruses

Stocks of the KOS and McKrae strains of HSV were grown and titrated in Vero cells (ATCC CCL81) and stored in aliquots at –80 °C. HSV KOS was concentrated from tissue culture grown virus by centrifugation and inactivated by treatment with β-propiolactone (BPL) (Li et al., 2006).

Infection and sampling

HSV KOS was used for footpad and intranasal infection, and HSV McKrae was used for intravaginal infection. Mice were anesthetized intraperitoneally with Avertin (2,2,2-tribromoethanol) prior to receiving 5×10^6 PFU of HSV i.n. (in 30 μl PBS), or 5×10^5 PFU of HSV (in 20 μl PBS) in each hind footpad. The intravaginal HSV dose was 1×10^6 PFU (in 20 μl PBS) given 5 days after subcutaneous injection (100 μl) of 2 mg/ml Depo-Provera (medroxyprogesterone acetate; Pharmacia and Upjohn, Kalamazoo, MI).

Anesthetized mice were exsanguinated via the retro-orbital plexus before tissue sampling. Tissues were processed to generate single-cell suspensions in IMDM (Invitrogen, Carlsbad, CA) containing L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), gentamicin (10 μg/ml), and 5×10^-5 M M β-mercaptoethanol (designated B cell medium, BCM), and supplemented with 10% fetal bovine serum (FBS). Lymph nodes [CLN, MLN, inguinal (IngLN), IliLN, and PLN] and spleen were collected and gently disrupted between the frosted ends of microscope slides. BM cell suspensions were obtained by flushing the femurs and tibiae. Red blood cells were removed from the spleen and BM preparations by ammonium chloride lysis. NALT was collected attached to the palate (Asanuma et al., 1997) and cells were released by teasing. PP were dissected from the small intestine and washed, and cells were released by digestion with 2 mg/ml
collagenase type I (Worthington, Lakewood, NJ) for 30 min at 37 °C. Washings from the vaginal tract were collected by flushing several times with 200 μl PBS. Sera and vaginal washings were stored at −80 °C.

ELISPOT assay

The ELISPOT assay was adapted to enumerate HSV-specific ASCs (Li et al., 2006). Nitrocellulose-bottomed 96-well Multiscreen HA filtration plates (Millipore, Bedford, MA) were coated with purified, detergent-disrupted HSV and blocked. Serial five-fold dilutions of single cell suspensions were prepared in BCM containing 10% FBS, and 100 μl volumes were added to the plates. Plates were incubated for 3–4 h at 37 °C in a humidified atmosphere containing 5% CO₂ and then washed thoroughly. Alkaline phosphatase (ALP)-conjugated isotype-specific goat anti-mouse Abs (Southern Biotechnology, Birmingham, AL) diluted 1:500 in PBS plus 5% BSA were added, and the plates were incubated overnight at 4 °C. After extensive washing, spots were developed at room temperature with 1 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO) in diethanolamine buffer. Plates were washed and dried after optimal spot development, and spots representing individual ASCs were counted using an Olympus SZX9 stereozoom microscope. The specificity of the assay was established by coating control plates with purified A/HKx31 (H3N2) influenza A virus.

Memory B cell assay

An LDA for determining HSV-specific MBC frequencies was used as described in detail previously. Briefly, two-fold dilutions of cells from immune mice were incubated in 96-well tissue culture plates, together with 10⁶ irradiated (3000 rad) syngeneic naïve spleen cell feeders plus BPL-inactivated HSV particles (0.01 μg/well). Plates were incubated for 4 days at 37 °C in a humidified atmosphere containing 5% CO₂. During this time, HSV-specific MBCs are stimulated to divide and differentiate into ASCs. After incubation, cells in each well were washed thoroughly and transferred to plates for the enumeration of HSV-specific ASCs by ELISPOT assay (described above). The ELISPOT plates were developed with ALP-conjugated goat anti-mouse IgG (Southern Biotechnology) to identify IgG ASCs. Pre-existing virus-specific ASC numbers in immune cell populations at the time of sampling were determined by direct ex vivo ELISPOT assay. After in vitro MBC activation and ELISPOT analysis, individual wells were scored positive for virus-specific MBCs if progeny ASC numbers were greater than the mean pre-existing ASC number plus 3 standard deviations. A minimum of 6 progeny ASCs were also required for a well to be scored positive. The virus-specific IgG MBC frequency was calculated from the number of negative wells per cell dilution by extrapolation to the dilution that gave 37% negative wells (Topham and Doherty, 1998). Virus-specific IgG MBCs are defined as cells that, upon in vitro stimulation, give rise to IgG ASCs (Coffman and Cohn, 1977; Okumura et al., 1976). At the time of sampling, immune cell populations were characterized by flow cytometry using FITC-conjugated anti-CD3ε (145-2C11) and PE-conjugated anti-B220 (RA3-6B2) mAbs (BD Biosciences) as staining reagents. To accommodate lymphoid tissue differences in the proportion of B220⁺ B cells, the population that includes the MBCs (Bell and Gray, 2003; Blink et al., 2005; Li et al., 2006), MBC frequencies are expressed for B220⁺ cells.

ELISA

Levels of HSV-specific Abs in serum and the vaginal wash were determined by ELISA using Costar immunoassay plates (Corning, Acton, MA) coated with purified, detergent-disrupted HSV (0.5 μg/well). Serial three-fold serum dilutions starting at 1:100, or a 1:20 dilution of the vaginal wash, were added to the plates. Otherwise, the ELISA was completed as described previously (Sangster et al., 2000) using ALP-conjugated goat anti-mouse IgG or IgA Abs (Southern Biotechnology) and p-nitrophenyl phosphate for detection of bound Abs. Absorbance at 405 nm was read using a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA). Ab concentrations in arbitrary units per milliliter were calculated from standard curves constructed using goat anti-mouse IgG or IgA capture Abs and purified mouse Ig standards (Southern Biotechnology).

Statistics

Statistical comparisons of mean values were performed using a two-tailed nonparametric Mann–Whitney U test for unpaired samples.

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References


