Mucosal and systemic antiviral antibodies in mice inoculated intravaginally with herpes simplex virus type 2

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Herpes simplex virus type 2 (HSV-2) causes lethal illness after intravaginal (IVAG) inoculation into BALB/cJ mice. In the present studies, we demonstrated in mice that primary IVAG vaccination with an attenuated strain of HSV-2 induced humoral immunity in sera and in vaginal secretions. Secondary genital exposure to HSV-2 enhanced this response. However, intraperitoneal exposure to attenuated HSV-2 elicited an antiviral antibody response in sera but not in vaginal secretions. In both sera and vaginal secretions, antiviral IgG antibodies were the major isotype. Systemic exposure to HSV-2 elicited antibodies only in sera that were specific for the major viral antigens whereas IVAG inoculation with HSV-2 stimulated both serum and vaginal antibody responses. Intravenous transfer of antiviral monoclonal antibodies protected against systemic HSV-2 infection but were ineffective against vaginal infection due to a lack of transudation into vaginal secretions. These results suggested that local humoral immunity in the genital tract is important in resistance to HSV-2.

Introduction

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen infecting the genital and oral mucosae (Corey & Spear, 1986; Rawls, 1985). After primary mucosal infection, HSV-2 can spread, in the absence of viraemia, to the central nervous system (CNS) and other organs. Recrudescent disease probably occurs by the activation of latent virus persisting in infected ganglia (Corey & Spear, 1986; Rawls, 1985; Walz et al., 1974). Although systemic vaccination protocols lack sufficient efficacy at present for use against mucosal HSV-2 infections in humans, several in vivo and in vitro studies indicate that immunity to or recovery from primary systemic infection by HSV-2 is mediated by humoral and cellular effector mechanisms. The roles of antibodies (Balachandran et al., 1982a; Morahan et al., 1977; Simmons & Nash, 1985), complement (Shore et al., 1979), interferon (Engler et al., 1981; Fitzgerald & Lopez, 1982), macrophages (Morahan et al., 1980; Plaeger-Marshall et al., 1983), cytotoxic T cells (CTLs) (Larsen et al., 1983; Rosenthal et al., 1987; Sethi et al., 1983), natural killer cells (Engler et al., 1981; Fitzgerald & Lopez, 1982), and cells involved in delayed hypersensitivity reactions (Nash & Ashford, 1982; Nash & Bell, 1983; Nash et al., 1981) and regulatory activities (Leung et al., 1984; Nash & Ashford, 1982) have all been implicated in resistance to systemic HSV-2 infection. In contrast, little is known about mucosal immune responses to HSV-2. Genital exposure to soluble (Batty & Warrack, 1955; Yang & Schumacher, 1979), cellular (Beer & Heaves, 1978; Chipperfield & Evans, 1972, 1975; Clarke, 1984; Kerr & Robertson, 1953; Waldman et al., 1972; Yang & Schumacher, 1979) and viral (Ogra & Ogra, 1973) antigens, including HSV-2 (Mendis et al., 1981; Merrinran et al., 1984; Nahmias et al., 1967), can result in specific antibody responses, including secretory IgA, in cervicovaginal secretions which may not be mirrored in the circulation. These observations correlate with increased numbers of plasmacytes in the cervicovaginal mucosa following exposure to antigen (Chipperfield & Evans, 1972). Also, the localized expression of cellular immune responses occurs in the genital tract (Clark & McDermott, 1981; Clark et al., 1983). Indeed, the mucosal immune response in the genital tract resembles that found at other mucosal sites (Bienenstock & Befus, 1980; McDermott et al., 1982) and is part of the mucosal immune system (McDermott & Bienenstock, 1979; McDermott et al., 1980; Weisz-Carrington et al., 1979). Thus, stimulation of mucosal immunity might have major importance in generating local resistance to HSV-2 and other pathogens. Studies of humoral immunity to microbial infections of the genital mucosa (Brunham et al., 1983; Chipperfield & Evans, 1972, 1975; Clarke, 1984; Kerr & Robertson, 1953; Levitt & Barol, 1987; Waldman et al., 1972; Widders et al., 1985;
Wilkie et al., 1972), support this hypothesis.

Previously we have shown (McDermott er al., 1984, 1987, 1989) that intravaginal (IVAG) inoculation with the attenuated ΔTK-HSV-2 strain (thymidine kinase-negative) elicited antiviral immunity in the genital tracts of mice. This response prevented the lethal spread of virulent HSV-2 from the vaginal mucosa to the CNS and was transferrable to recipient mice with genital lymph node (GLN) T cells but not with sera from inoculated animals or cells from other anatomical sites. These findings suggested that either IVAG inoculation with ΔTK-HSV-2 failed to induce sufficient antiviral antibodies in sera and vaginal secretions or that such antibodies could not protect against mucosal challenge by virulent HSV-2.

In the present studies, we examined the antiviral antibodies in sera and vaginal washings obtained from mice following IVAG systemic exposure to HSV-2. The results indicated that genital infection by HSV-2 elicits virus-specific antibody responses in sera and vaginal washings although such antibodies in sera do not enter genital secretions.

Methods

Virus propagation. Wild-type (TK⁺) HSV-2 strain 333 and ΔTK HSV-2 were propagated and stored until used as described previously (McDermott et al., 1984, 1987).

Inoculation of mice with HSV-2. Female BALB/cJ (HRI) mice (Health Research Incorporated) were used at 6 to 8 weeks of age (McDermott et al., 1984, 1987). Mice were inoculated IVAG, intraperitoneally (i.p.) or in the footpads (f.p.) with HSV-2 suspended in 10, 250 or 10 µl, respectively, of phosphate-buffered saline (PBS). Mice were individually coded by ear punch and examined daily for genital secretions.

Collection of sera and vaginal washings. Sera were obtained via cardiac exsanguination or the retro-orbital plexus. Vaginal washings were prepared similarly. Mucus and particulate matter in the washings were removed by centrifugation. Sera and washings were stored at -70 °C until used.

ELISA. An ELISA was used to detect and quantify anti-HSV-2 antibodies in sera and vaginal washings. As it was not possible technically to measure antiviral antibodies in the small volumes of vaginal washings obtained from individual mice, washings from animals within a group were pooled for analyses. Sera were analysed individually and then as group pools.

A virus-infected cell lysate was prepared by inoculating Vero cell monolayers with TK⁺ HSV-2 at an m.o.i. of 10, harvesting and washing the 2 × 10⁶ cells 18 to 24 h later with PBS and lysing the cells in PBS by freezing and thawing followed by sonication and high-speed centrifugation to remove cell debris. Uninfected Vero cell lysates were prepared similarly. Microtitre plate wells (Immunlon 2; Dynatech Laboratories) were incubated with 100 µl of the virus-infected or uninfected cell lysate (100 µg total protein in PBS containing 200 µM-PMSF) for 2 h at 37 °C. The wells were washed with 0.05% Tween 20 in 0.02 M-Tris hydroxymethylaminomethane (HEAM)-HCl pH 7.4, containing 0.15 M-NaCl and 0.005 M-KCl (referred to as Tris-T) and incubated for 30 min at 37 °C with 150 µl of 0.1% bovine serum albumin in 0.02 M-Tris HEAM-HCl-buffered saline pH 7.4 (referred to as the detergent). After washing with Tris-T, the wells were incubated for 1 h at 37 °C with 100 µl of sera or vaginal washings diluted in diluent. Wells were washed with Tris-T and 100 µl of alkaline phosphatase-conjugated goat anti-mouse Ig (heavy chain-specific; Southern Biotechnology Associates) diluted in diluent (1/125 for anti-IgA or anti-IgG; 1/500 for anti-IgM) was added to each well for 1 h at 37 °C. The wells were washed with Tris-T and 100 µl of 1.0 M-diethanolamine buffer pH 9.8, containing 0.05 M-MgCl₂ and 1.0 mg/ml p-nitrophenyl phosphate (5 mg phosphatase substrate tablets; Sigma) was added to each well. After a 30 min incubation at 37 °C, the absorbance of the solution in each well was determined at 410 nm using a spectrophotometer (Dynatech Laboratories). The absorbance determinations revealed that compared to the buffer system alone, antiviral antibodies did not bind to wells coated with uninfected Vero cell lysates nor did normal mouse sera bind to wells coated with HSV-2-infected or uninfected Vero cell lysates. Thus, uninfected Vero cell lysates were used to establish baseline absorbance values. The absorbance measurements were related to the baseline values and the concentrations of anti-HSV-2 antibody isotypes were estimated by comparison with calibration curves prepared by reacting dinitrophenylated (DNP-treated) human serum albumin-coated microtitre plates (Ninno et al., 1984) with known amounts of purified anti-DNP-specific IgM, IgG or IgA antibodies produced by MOPC-774, MPC-11 or MOPC-460 murine myeloma cells, respectively. The assays used solid-phase antigen excess with the minimum sensitivities estimated from the calibration curves at 16, 32 and 62 ng/ml of antigen-specific IgM, IgG or IgA, respectively.

Viral glycoprotein specificity. The viral glycoprotein specificity of the anti-HSV-2 immunoglobulin in sera or vaginal washings was assessed by immunoprecipitation methods (Johnson & Feenstra, 1987). Briefly, TK⁺ HSV-2-infected Vero cells were labelled with [³⁵S]methionine between 3 and 8 h post-infection (p.i.), a time at which all host protein synthesis had halted as a result of HSV-2 infection (Spear, 1984). Uninfected Vero cells were labelled similarly. An extract of the cells was mixed with sera or vaginal washings prepared from virus-inoculated mice. To ensure the recovery of the small amounts of antibodies detected in vaginal washings, 5 µl of an anti-Ig reagent was added 0.75 h later to the cell lysates or vaginal washings; this reagent was prepared by combining equal amounts, in terms of protein concentration, of rabbit anti-mouse IgM, IgG and IgA sera (Zymed Laboratories). The antigen–antibody complexes were precipitated with Protein A-Sepharose, eluted and electrophoresed in SDS-polyacrylamide gels (Laemmli, 1970). Monoclonal antibodies (MAbs) having defined specificities for HSV-2 glycoproteins gD (clone 18/β3), gC (clone 17/α2) or gB (clone 15/β2) (Balachandran et al., 1984a, b) were used as controls to identify the presence of selected viral antigens. Mₐ markers were included on the same gels. After fluorography and drying, the gels were exposed to X-ray film and developed.

Passive immunization. Mice were injected i.p. or intravenously (i.v.) with 500 µl of PBS or ascites fluid produced by hybridoma cells secreting MAbs specific for HSV-2 glycoproteins gD (clone 18/β3), gC (clone 17/α2) or gB (clone 15/β2) (Balachandran et al., 1984a, b; Ligas & Johnson, 1988). Mice were inoculated, 3 h after receiving antibodies, via the i.p., f.p. or IVAG routes with 1 × 10⁶ f.p.u. of TK⁺ HSV-2 and examined daily thereafter.
Results

Detection of anti-HSV-2 antibodies

Our previous studies (McDermott et al., 1984, 1987, 1989) showed that IVAG inoculation of mice with ΔTK−HSV-2 induced immunity, within 7 days, to lethal IVAG challenge with TK+ HSV-2. Such immunity was attributable solely to GLN T cells. To determine whether an absence of virus-specific antibodies in serum might have been responsible for this finding, studies using indirect immunofluorescence on virus-infected cells were conducted. These studies revealed that 1 week after a primary IVAG infection by ΔTK−HSV-2, antiviral antibodies were present in sera and vaginal washings (data not shown). Table 1 confirms and extends these findings. Primary IVAG inoculation with ΔTK−HSV-2 elicited virus-specific antibodies in pooled sera, and to a lesser extent, in pooled vaginal washings, which was virtually all of the IgG isotype; IgM and IgA virus-specific antibody concentrations in these fluids were not above the detection limits of the assay. IVAG challenge with ΔTK− or TK+ HSV-2 enhanced the IgG response in sera but not in vaginal washings. However primary infection and challenge with ΔTK−HSV-2 via the i.p. route produced a lesser antiviral IgG antibody response in sera but not in vaginal washings. Several experiments confirmed these findings as did the evaluation of individual serum samples. The results demonstrated that IVAG infection by ΔTK−HSV-2 elicited antiviral humoral immunity in sera and vaginal washings which was improved by re-exposure to ΔTK− or TK+ HSV-2. Furthermore, systemic inoculation with ΔTK−HSV-2 appeared to be an inappropriate route for eliciting antiviral humoral immunity in the genital tract.

Antigen specificity of antiviral immunoglobulins

The antigen specificity of antiviral antibodies present in sera and vaginal washings obtained from HSV-2-infected mice was determined by immunoprecipitation studies. Fig. 1 shows that sera or vaginal washings taken from mice inoculated IVAG with PBS did not contain antibodies capable of precipitating HSV-2 polypeptides (lanes 1 and 6). Similarly, sera obtained from mice that were inoculated and challenged IVAG or i.p. with virus did not contain antibodies which precipitated polypeptides prepared from uninfected Vero cells (lanes 13 and 14). Antibodies in sera obtained from HSV-2-infected mice precipitated several polypeptides, including those with electrophoretic migration characteristics and Mr, values typical of HSV-2 glycoproteins gB and gC (lanes 2 to 5 and 11 and 12) (Balachandran et al., 1982a, b; Spear, 1984). Although the challenge regimens resulted in antiviral, serum-derived antibody responses which paralleled those presented in Table 1, as judged by the amounts of virus-associated radioactivity precipitated prior to electrophoresis (data not shown), none of these regimens altered the serum-derived, antiviral polypeptide specificities of the antibodies.

In vaginal washings obtained from mice inoculated via the IVAG route, the polypeptide specificities of the antiviral antibodies were identical to those observed in sera (lanes 7 to 9). However despite the fact that mice inoculated i.p. possessed HSV-2 polypeptide-specific antibodies in their sera (lanes 2 to 5), such antibodies were significantly reduced in vaginal washings; only a very faint triad of specific bands consistent with the location of glycoprotein gB were noted (lane 10). The results of the immunoprecipitation analysis were confirmed by several experiments. When taken together with the data presented in Table 1, the results suggested that IVAG or i.p. inoculation and challenge with ΔTK− or TK+ HSV-2 elicited responses in sera that differed only quantitatively from those in vaginal washings. These findings suggested also that the antiviral IgG antibodies found in vaginal washings following mucosal exposure to HSV-2 probably resulted from local synthesis and not from appreciable serum transudation into the vaginal lumen.

Passive immunization by antiviral MAbs

The presence of HSV-2-specific antibodies in sera but not in vaginal washings obtained from mice inoculated...
i.p. with HSV-2 suggested that humoral immunity to the virus in the genital tract might be mucosal in origin. Thus, systemic transfer of MAbs specific for HSV-2 glycoproteins, which will protect mice against systemic HSV-2 infection (Balachandran et al., 1982a), might not protect against IVAG challenge with TK+ HSV-2. The results depicted in Fig. 2 support this hypothesis. Mice injected i.v. with ascites fluids containing IgG MAbs specific for glycoproteins gC or gD resisted a lethal systemic inoculation with TK+ HSV-2 as compared to animals injected with PBS. No differences in these results were noted when the MAbs were administered in ascites fluids via the i.p. route (data not shown). In contrast, similarly treated mice did not resist lethal IVAG inoculations with TK+ HSV-2. Several experiments confirmed these findings. Further confirmatory results were obtained after i.v. injection of mice with 10 mg of each MAb that had been purified by Protein A-Sepharose affinity chromatography followed by IVAG or i.p. challenge with the virus (data not shown). Thus, circulating antiviral antibodies were not capable of protecting mice from a lethal IVAG HSV-2 infection. Studies using immunoprecipitation methods were conducted to assess whether the circulating, virus-specific MAbs reached the vaginal mucosa. Fig. 3 shows that sera and vaginal washings taken from mice 24 h after i.v. injection with PBS did not contain antibodies capable of precipitating HSV-2 glycoproteins (lanes 1
and 5) nor did these fluids contain antibodies which reacted with uninfected Vero cell lysates (data not shown). However, HSV-2 glycoprotein-specific antibodies were readily detectable in the sera of mice injected 24 h previously with glycoprotein gB-specific MAbs (lane 2), but such antibodies were not detected in vaginal washings taken from the same animals (lane 6). This latter observation was not attributable to the sensitivity of the assay since the glycoprotein gB-specific MAbs, which have exceptional antigen precipitation characteristics in the assay (Ligas & Johnson, 1988), were clearly detectable if the genital tracts of passively immunized mice had been inflamed by IVAG challenge with TK⁺ HSV-2 (data not shown). These findings were confirmed by an independent experiment. The results demonstrated that serum-borne IgG MAbs specific for HSV-2 glycoproteins did not transude into the vaginal secretions of healthy mice.
Discussion

In this report, we demonstrated IgG antibodies specific for HSV-2 glycoproteins in sera and vaginal secretions (concomitantly with cellular immunity in GLN (McDermott et al., 1989) following IVAG or systemic inoculation and challenge with HSV-2. Furthermore, the results indicated that the antiviral antibody responses detected in vaginal secretions were probably derived locally and that serum-borne IgG antibodies did not enter the genital mucosa. These findings suggested that antigenic stimulation of the humoral elements of the mucosal immune system may be important for resistance to genital pathogens.

The predominance of IgG antibodies in sera obtained 2 weeks after primary i.p. or IVAG inoculation with ΔTK−HSV-2 indicated that infection by this virus provoked a progression of immunity that was typical of a transition in synthesis from IgM to IgG. Regardless of the secondary challenge regimen, IgG remained the predominant isotype in sera and vaginal washings. It is not surprising that antiviral IgA antibodies were not found in sera after systemic inoculation with HSV-2 since the precursors of IgA plasmacytes are rare in murine peripheral lymphoid tissues (McDermott et al., 1982). In systemically HSV-2-infected mice, the transient induction of anti-HSV-2 IgM antibodies has been noted together with the continuous synthesis of IgG antibodies (Rawls, 1985). Furthermore, in these studies the route of inoculation was important since i.p. inoculation produced greater antiviral serum antibody responses than did subcutaneous inoculation. In the present study, exposure of the genital mucosa to HSV-2 led to antiviral humoral responses in sera that were substantially greater than those found after an i.p. inoculation. This may reflect the virulence of HSV-2 at a given anatomical site and the subsequent availability of viral antigens to stimulate the relevant lymphoid tissues.

In contrast, primary IVAG inoculation with ΔTK−HSV-2 elicited an antiviral IgG response in vaginal washings that was appreciably less than that found in sera. Furthermore, no antiviral IgM or IgA was detected in these fluids after a primary IVAG or i.p. infection and challenge with HSV-2. Presumably, anti-HSV-2 serum responses were generated within the GLNs and by the dissemination of plasmablasts to distant sites (McDermott & Bienenstock, 1979; McDermott et al., 1980). Moreover, smaller antibody responses in vaginal washings compared to sera may reflect the small numbers of plasmacytes present in the murine genital tract (McDermott & Bienenstock, 1979; McDermott et al., 1980; Parr & Parr, 1985). Studies to identify the anatomical locations of HSV-2-specific plasma cells are in progress.

Disparities between serum and secretory humoral immune responses are well recognized following mucosal exposure to antigens (Batty & Warrack, 1955; Bienenstock & Befus, 1980; Kerr & Robertson, 1953; Levitt & Barol, 1987; McDermott et al., 1980, 1982; McDermott & Bienenstock, 1979; Ogra & Ogra, 1973; Weisz-Carrington et al., 1979). Generally, an antigen-specific IgA response in external secretions is the hallmark of mucosal immune responsiveness, although this isotype often occurs together with an IgG response in the sera. It is not known why IgG was the predominant anti-HSV-2 isotype measured in vaginal washings although it was likely not to be of serum origin. IgG-containing plasmacytes are found in the genital mucosa of mice (McDermott & Bienenstock, 1979; McDermott et al., 1980; Parr & Parr, 1985) and, certainly, the local production of antigen-specific IgA and IgG antibodies secreted into cervico-vaginal fluids of women with genital HSV-2 (Mendis et al., 1981; Merrinran et al., 1984) or Chlamydia trachomatis infections (Brunham et al., 1983; Levitt & Barol, 1987; Richmond et al., 1980) suggests that both of these isotypes are important in controlling the magnitude and duration of these sexually transmitted diseases.

Several virion-encoded glycoproteins, including gC, gB, gD, gE and gG, displayed on the envelope of HSV-2 and on the surface of HSV-2-infected cells are determinants of viral pathogenicity (Spear, 1984). Following systemic inoculation with HSV-2 or its subunits, antibodies specific for virion-encoded glycoproteins were detected in human and murine sera (Ashley et al., 1985; Balachandran et al., 1982a, b; Long et al., 1984; Spear, 1984). In the present studies, IVAG inoculation and challenge with HSV-2 elicited similar antibody responses to virus-encoded glycoproteins in both sera and vaginal washings. Glycoproteins gB, gC and gD are known to induce neutralizing and complement-dependent cytolytic antibodies (Balachandran et al., 1982b; Corey & Spear, 1986; Rawls, 1985; Spear, 1984) whereas gB and gC also elicit delayed hypersensitivity responses (Corey & Spear, 1986; Rawls, 1985) and act as recognition elements for major histocompatibility complex class I-restricted CTLs (Blacklaws et al., 1987; Corey & Spear, 1986; Rawls, 1985; Rosenthal et al., 1987). These findings indicate that the mechanisms responsible for the stimulation of antiviral humoral immunity are similar at systemic and mucosal sites and that analogous recognition of viral antigens at distant mucosal sites might be expected.

Passive immunization with polyclonal anti-HSV-2 sera or MAbs specific for HSV-2 glycoproteins (Balachandran et al., 1982a; Corey & Spear, 1986; Rawls, 1985; Simons & Nash, 1985) as well as active systemic immunization with glycoprotein gD (Berman et al., 1985; Cremer et al., 1985; Long et al., 1984) protected
murine species from systemic challenge with HSV-2. This method of defining the antigenic content of a systemically administered viral subunit vaccine has been extended to humans and has shown promise in eliciting neutralizing serum antibodies specific for gB, gC and gD (Ashley et al., 1985). However, the present studies suggest that serum antibodies may not play a major role in the defence against a genital HSV-2 infection. Virtually all of the antiviral antibodies in mouse vaginal washings appeared to be produced locally and, indeed, passive transfer of MAbS that protected against lethal systemic HSV-2 infection failed to enter vaginal secretions and confer protective immunity against a lethal IVAG inoculation. Similarly, large amounts of antisera transferred i.v. did not protect mice from primary intrauterine infection with C. trachomatis (Levitt & Barol, 1987; Tuffrey et al., 1985). In fact, very little serum-borne IgG crosses healthy mucosae into secretions (Bienenstock & Befus, 1980; McDermott et al., 1982).

When taken together, the present findings were consistent with the observation that most antibodies found in cervico-vaginal secretions are synthesized by local plasma cells (Bienenstock & Befus, 1980; Chipperfield & Evans, 1972, 1975; McDermott et al., 1980, 1982; McDermott & Bienenstock, 1979; Wilkie et al., 1972). Thus, vaccination protocols inducing only systemic humoral responses to HSV-2 may not be valuable in eliciting anti-HSV-2 antibodies in vaginal secretions. Such antibodies may be required to prevent virus absorption to host cells and to inhibit significant virus dissemination until an appropriate cellular response can develop (McDermott et al., 1989).

Previously, we have shown that the gut-associated lymphoid tissue is an enriched source of IgA and IgG plasmacyte progenitors found in the genital mucosa (McDermott & Bienenstock, 1979; McDermott et al., 1980) and that the mucosal immune response in the genital tract is influenced by sex hormones (McDermott et al., 1980; Wira & Sandoe, 1977). The observation that immunity in the genital mucosa is stimulated by replicating antigens in a manner similar to that noted at other mucosal sites (Bienenstock & Befus, 1980; McDermott et al., 1982), particularly in the intestine, supports the hypothesis that genital immunity can be engendered also by an anatomically distant stimulation of the common mucosal immune system (Bienenstock & Befus, 1980; McDermott et al., 1980, 1982; McDermott & Bienenstock, 1979; Weisz-Carrington et al., 1979) and/or perhaps further enhanced by systemic immunization. Such knowledge will be valuable for understanding and manipulating host resistance at the genital mucosal surface to a variety of pathogens.

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