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Immunity to vaginal HSV-2 infection in immunoglobulin A knockout mice

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SUMMARY

An immunoglobulin A (IgA) knockout (KO) mouse was used to study the role of IgA in protective immunity against vaginal infection with herpes simplex virus-type 2 (HSV-2). Intact and KO mice were immunized intravaginally (IVAG) with attenuated HSV-2, challenged IVAG with wild-type virus 6 weeks later and evaluated for vaginal infection and neurological disease. Non-immunized/challenged intact and KO mice showed vaginal infection and succumbed to neurological disease, while immunized/challenged mice exhibited reduced or no vaginal infection and no neurological disease. Log 2·5 enzyme-linked immunoassay (ELISA) titres of viral IgA, immunoglobulin G (IgG) and immunoglobulin M (IgM) in vaginal secretions collected from intact immune mice before challenge were $0{\cdot}6 \pm 0{\cdot}3$, $6{\cdot}4 \pm 0{\cdot}32$ and 0·0, while those in KO immune mice were 0·0, $6{\cdot}7 \pm 0{\cdot}19$ and $3{\cdot}0 \pm 0{\cdot}29$, respectively. Twenty-four hours after challenge, the percentage of vaginal epithelium that was infected in non-immune intact and KO mice was $2{\cdot}0 \pm 0{\cdot}6$ and $2{\cdot}4 \pm 0{\cdot}6$, which was reduced to $0{\cdot}2 \pm 0{\cdot}1$ and $0{\cdot}1 \pm 0{\cdot}06$ in immune intact and KO mice, respectively. No shed virus protein was detected in vaginal secretions 3 days after challenge in any immune mouse, whereas titres were 1400 and 1700 in the two groups of non-immune mice. Thus, immune protection against vaginal HSV-2 infection was similar in both KO and intact mice, indicating that this mucosal immunity does not depend mainly on IgA.

INTRODUCTION

Local immunization at mucosal surfaces often elicits mainly secretory immunoglobulin A (S-IgA) antibody, which plays the major role in immune protection at these mucosal surfaces.[1–3] Studies performed in humans and animal models have demonstrated that the level of immune protection against infections of the intestinal or upper respiratory tracts correlates better with the level of antibodies in corresponding external secretions than in serum.[4–7] S-IgA present at mucosal surfaces is mainly produced locally (by plasma cells distributed in mucosal tissues) and is selectively transported by a receptor-mediated pathway through epithelial cells into external secretions. S-IgA resists proteolytic cleavage,[3,8] neutralizes pathogens at the mucosal surface[3] and forms intracellular complexes with viruses to inhibit virus replication and to prevent or resolve infections.[9]

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Abbreviations: DP, Depo-Provera®; E, oestradiol benzoate; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; HSV-2, herpes simplex virus-type 2; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IVAG, intravaginal; KO, knockout; PBS, phosphate-buffered saline; PFU, plaque-forming units; S-IgA, secretory immunoglobulin A; s.c., subcutaneous; TK, thymidine kinase.

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It is often assumed that S-IgA is the main protective antibody at mucosal surfaces of the female genital tract and that induction of a S-IgA response will be required to produce optimal immunity in this location (see cross-references in reference 10). However, direct evidence supporting this assumption is lacking. Recently, Parr & Parr[11] showed that IgG is the main protective antibody in mouse vaginal secretions following local immunization with attenuated herpes simplex virus-type 2 (HSV-2). Unfractionated vaginal immunoglobulins, from immune and non-immune mice, and affinity-purified immunoglobulin G (IgG) and S-IgA from immune secretions were incubated (at their *in situ* concentrations in vaginal mucus) with wild-type HSV-2 *in vitro*, followed by inoculation into vaginae of non-immune mice. HSV-2 was neutralized by unfractionated antibody and purified IgG from immune secretions, but not by unfractionated non-immune antibody or by purified immune S-IgA. The protective effect of IgG *in vivo* was investigated by passively transferring purified serum IgG from immune and non-immune donors to non-immune recipients before vaginal challenge infection. Immune IgG significantly reduced the percentage of vaginal epithelium infected, shed virus protein concentrations in the vaginal lumen and illness scores, despite the viral antibody titres in serum and vaginal secretions of recipient mice at the time of challenge being only 29% and 8%, respectively, of those in actively immunized mice. Collectively, the data indicated that IgG antibody in vaginal secretions of immune mice provided early protection against vaginal challenge infection

with HSV-2 infection, probably by neutralizing virus in the vaginal lumen. In contrast, S-IgA antibody contributed relatively little to immune protection of the vagina.

The purpose of the experiments described here was to investigate the effects of S-IgA deficiency on immune protection against vaginal HSV-2 challenge in an IgA knockout (KO) mouse that was developed by deleting the IgA switch and constant regions through gene targeting.[12] Mice homozygous for this deletion developed normally and demonstrated no increased susceptibility to infections under conventional housing conditions and lacked detectable IgA in gastrointestinal, nasal and pulmonary secretions.

MATERIALS AND METHODS

Virus and animals

Wild-type HSV-2 and attenuated HSV-2 (a strain that contains a partial deletion of the thymidine kinase gene), HSV-2-infected Vero cell lysates and uninfected Vero cell lysates were generously provided by Dr Mark McDermott, McMaster University, Hamilton, Canada.[13,14]

Breeding pairs of 129 SV X C57BL/6 intact and IgA-KO mice were provided by Dr Gregory Harriman. The mice were housed and bred in the SIUC Vivarium in compliance with all institutional and federal animal welfare requirements, and all experimental procedures were approved by the institutional Animal Care and Use Committee. The 60 intact and 60 IgA-KO offspring used in these studies were 10–18 weeks old at the start of treatment.

Hormonal treatment and immunization

In the first experiment, 20 intact and 20 KO age-matched mice were injected subcutaneously (s.c.) with 0·10 μg oestradiol benzoate (E) in peanut oil, followed 24 hr later by s.c. injection of 2·0 mg Depo-Provera® (DP, Upjohn Co., Kalamazoo, MI) diluted in phosphate-buffered saline (PBS) (E/DP-treated mice).[15] Five days after treatment with DP, 15 of the mice in each group were anaesthetized with tribromoethanol and immunized by intravaginal (IVAG) inoculation with 20 μl of attenuated HSV-2 at $3{\cdot}5 \times 10^6$ plaque-forming units/ml (PFU/ml); the remaining mice were not immunized. These animals are referred to as immune and non-immune mice, respectively. All mice were retreated with E/DP after 5 weeks and vaginal washes were collected once daily from 5 to 7 days later for evaluation of S-IgA concentration and antiviral antibody titres. Two days after the last vaginal wash, all of the mice were challenged intravaginally with 20 μl of wild-type HSV-2 ($4{\cdot}2 \times 10^6$ PFU/ml). Vaginal washes were collected again, 3 days after challenge, for evaluation of shed virus protein titres. All mice were examined daily for signs of illness 8–14 days after challenge. Illness was indicated by ruffled fur, arched backs, feeble movements, swollen vulva and paralysis of one or both hindlimbs.

In the second experiment, 30 intact and 30 KO age-matched mice were treated with E/DP as described above. Twenty mice from each group were inoculated with attenuated virus and the remaining 10 mice in each group were left untreated. After 6 weeks the mice were retreated with E/DP and vaginal washes were collected once daily 5–7 days later for evaluation of S-IgA concentration and antiviral antibody titres. Ten immunized and 10 non-immunized mice in each group were then challenged with wild-type virus. These mice were killed 24 hr after challenge, and the vaginae were removed, fixed (at 4 for 2 hr) with 2% paraformaldehyde in 0·1 M phosphate buffer, pH 7·4, frozen and stored at −70 until required for evaluation of epithelial infection.[15] Ten intact and 10 IgA KO mice that were immunized with attenuated virus were not challenged with wild-type virus. The vaginae of these mice were collected at the same time as those from the challenged mice and assessed for histological evidence of pre-existing inflammation/infection.

Vaginal secretions

Immunoglobulins were extracted from vaginal secretions, and viral IgG and S-IgA antibody titres were measured by enzyme-linked immunosorbent assay (ELISA), as previously described.[11,16] The titre was defined as the reciprocal of the sample dilution at which the absorbance was reduced to 0·30 above the background absorbance of non-immune samples at the same dilution (0·05 or less). Viral immunoglobulin M (IgM) antibody was measured similarly except that a mixture of recombinant HSV glycoprotein B and glycoprotein D (Austral Biologicals, San Ramon, CA) was used as antigen because high background values were encountered when HSV-2-infected Vero cell lysate was used as antigen. The concentration of IgA in vaginal secretions was measured by ELISA, as previously described,[16] using a mouse S-IgA standard purified from milk.[17] The titres of shed virus proteins were measured by ELISA in extracts of vaginal secretions that were collected 72 hr after vaginal challenge with wild-type virus, as previously described.[11] Titre was defined as the reciprocal of the dilution at which the absorbance was reduced to 0·30 above the background absorbance of wells that contained vaginal washes from immune mice that were not challenged with HSV-2.

Immunolabelling of HSV-2, lymphocytes and plasma cells in the vagina

Immunolabelling of HSV-2 in the vagina and quantification of epithelial infection were carried out as previously described.[15,18] Briefly, cryostat sections of vagina were immunolabelled using fluorescein isothiocyanate (FITC)-rabbit anti-HSV-2 (Dako, Carpinteria, CA). The percentage of vaginal epithelium infected with HSV-2 was determined by using an image analysis system. The lengths of HSV-2-labelled segments and total lengths of vaginal epithelium were measured in histological sections sampled from four areas of each vagina. The mean percentage of HSV-2-infected epithelium in each group was calculated and statistical analysis was performed using the Mann–Whitney *U*-test.

Immunolabelling and quantification of vaginal $CD4^+$, $CD8^+$ and $B220^+$ lymphocytes, and IgA, IgG and IgM plasma cells were performed as previously described.[19] To quantify plasma cells, cryostat sections were postfixed for 10 min in acetone, blocked for 30 min in 2% goat serum and incubated for 1 hr with one of the following antibodies: TRITC rabbit anti-mouse IgA (α), FITC rabbit anti-mouse IgG (γ) or FITC rabbit anti-mouse IgM (μ) (Jackson Immunoresearch Laboratories, West Grove, PA). For controls, TRITC or FITC rabbit IgG was used in place of the primary antibodies.

RESULTS

Measurement of IgA and anti-HSV-2 antibodies in vaginal fluids

The concentrations of IgA and the antiviral antibody titres in vaginal secretions of non-immune and immune intact and KO mice are shown in Table 1. The KO mice had no detectable IgA in vaginal secretions while the concentration of IgA in non-immune and immune intact mice was $20{\cdot}0 \pm 5{\cdot}1$ and $18{\cdot}0 \pm 3{\cdot}5$ μg/ml, respectively. The vaginal extracts were ≈20-fold dilutions of vaginal mucus.[16] Immune KO mice had no detectable virus-specific IgA antibodies in vaginal secretions while trace amounts were detected in immune intact mice. Both intact and KO immune mice had specific IgG viral antibodies in vaginal secretions and the log 2·5 geometric mean titres were not significantly different ($P = 0{\cdot}47$). Specific IgM antibodies were present only in immune KO mice. Comparable results were obtained in a second experiment (data not shown).

Effects of immunization on vaginal infection and neurological disease

The effects of IVAG immunization with attenuated HSV-2 on vaginal epithelial infection, shed virus protein titres and neurological disease in intact and KO mice are summarized in Table 2. Twenty-four hours after challenge, non-immunized intact and KO mice showed labelling of HSV-2 in the vaginal epithelium in eight of 10 mice in each group and in $2{\cdot}0 \pm 0{\cdot}6\%$ and $2{\cdot}4 \pm 0{\cdot}6\%$ of the epithelium, respectively (Figs 1 and 2). Three days after challenge, shed virus protein was detected in all non-immune mice, and all non-immunized mice succumbed to neurological disease, by 9 days after vaginal challenge. In contrast, immunized mice at 24 hr after challenge showed only four of 10 mice and $0{\cdot}2 \pm 0{\cdot}1\%$ of the epithelium infected or three of 10 mice and $0{\cdot}1 \pm 0{\cdot}06\%$ epithelial infection in intact and KO mice, respectively. These differences were not statistically significant. No shed virus protein was detectable in vaginal secretions from the immune mice 3 days after challenge and none of the immune mice showed any signs of neurological disease 8–14 days after challenge.

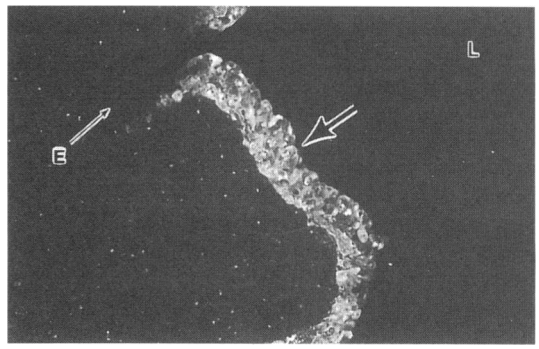

Figure 1. Fluorescence micrograph (magnification ×250) showing immunolabelling of HSV-2 (large arrow) in a section of the vaginal epithelium of a non-immune mouse 1 day after challenge with wild-type virus. Adjacent regions of the epithelium (E) were negative. Fluorescence in the stroma is caused by endogenous fluorescence of granulocytes. L, lumen.

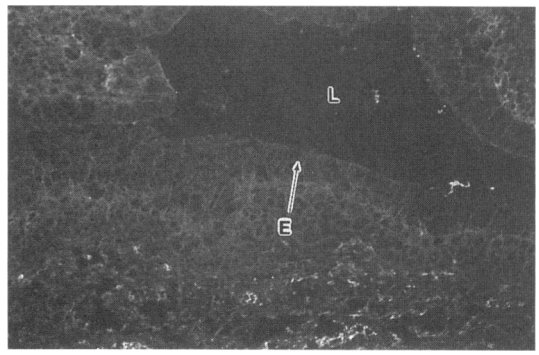

Figure 2. Fluorescence micrograph (magnification ×250) showing no labelling of HSV-2 in the vaginal epithelium (E) when FITC-conjugated normal rabbit IgG replaced FITC-conjugated rabbit anti-HSV-2. L, lumen.

Immune cells in the vagina

Immunolabelling of IgA, IgG, and IgM in vaginae from intact immune mice showed a few IgA plasma cells and a greater number of IgG and IgM plasma cells, while non-immune mice of this strain showed few plasma cells of any isotype. Non-

Table 1. Concentration of immunoglobulin A (IgA) and enzyme-linked immunosorbent assay (ELISA) titres of specific viral antibodies in vaginal secretions of intact and knockout (KO) mice.

Mice	Immunity*	IgA† (μg/ml)	Anti-HSV-2‡ IgA	IgG	IgM
Intact	Non-immune	$20{\cdot}0 \pm 5{\cdot}1$	0 (1·0)	0 (1·0)	0 (1·0)
KO	Non-immune	0	0 (1·0)	0 (1·0)	0 (1·0)
Intact	Immune	$18{\cdot}0 \pm 3{\cdot}5$	$0{\cdot}6 \pm 0{\cdot}3$ (1·7)	$6{\cdot}4 \pm 0{\cdot}32$ (360)	0 (1·0)
KO	Immune	0	0 (1·0)	$6{\cdot}7 \pm 0{\cdot}19$ (480)	$3{\cdot}0 \pm 0{\cdot}29$ (16)

*Immune mice were inoculated with attenuated virus 6 weeks before collection of vaginal secretions.

†Mean ± SEM.

‡Log 2·5 geometric mean titres ± SEM are followed in parenthesis by the geometric mean titres.

Table 2. The effects of immunization on vaginal epithelial infection, shed virus protein concentrations and neurological disease in intact and knockout (KO) mice.

Mice	Immunity*	Mean % epithelial infection ± SEM	Shed virus protein§	Mice showing neurological disease
Intact	Non-immune	2·0 ± 0·6 8/10¶	6·6 ± 0·6 (1400) 5/5¶	5/5
KO	Non-immune	2·4 ± 0·6 8/10¶	6·7 ± 0·5 (1600) 4/4¶	4/4
Intact	Immune	0·2 ± 0·1† 4/10¶	0·0 (1·0) 0/14¶	0/14
KO	Immune	0·1 ± 0·06‡ 3/10¶	0·0 (1·0) 0/14¶	0/13

*Immune mice were inoculated with attenuated virus 6 weeks before vaginal challenge with wild-type HSV-2.

†Significantly less by comparison with intact non-immune mice ($P = 0{\cdot}0115$; Mann–Whitney *U*-test).

‡Significantly less by comparison with non-immune KO mice ($P = 0{\cdot}0052$; Mann–Whitney *U*-test), but not significantly different from intact immune mice ($P = 0{\cdot}58$; Mann–Whitney *U*-test).

§Log_3 geometric mean titres ± SEM followed in parenthesis by the geometric mean titres.

¶No. of mice infected/total no. of mice.

immune and immune KO mice showed many plasma cells of the IgM isotype, fewer of the IgG isotype and an absence of IgA plasma cells. The numbers of $CD4^+$, $CD8^+$ and $B220^+$ lymphocytes in vaginae from non-immune and immune intact and KO mice are shown in Fig. 3. Few lymphocytes were detected in the vaginae of non-immune mice, but greater numbers were present in immune mice. There were no significant differences in the numbers of $CD4^+$ and $CD8^+$ lymphocytes in intact and KO immune mice, but the numbers of $B220^+$ lymphocytes were markedly lower in KO mice ($P = 0{\cdot}02$).

Susceptibility to vaginal infections

Histological sections of vaginae from 10 intact and 10 KO mice, immunized with the attenuated virus 6 weeks earlier, were stained with haematoxylin and eosin and examined for the presence of inflammatory cells, such as neutrophils and lymphocytes, which were identified on their histological appearance. Few neutrophils or lymphocytes were found in any of the 20 vaginae examined. Thus, there was no indication that our results in KO mice were compromised by vaginal infections at the time of HSV-2 challenge.

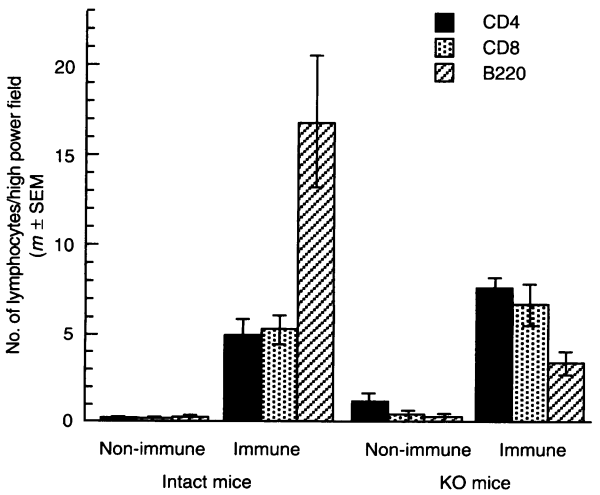

Figure 3. Lymphocytes (CD4, CD8 and B220) present in vaginal mucosa 24 hr after vaginal challenge with wild-type HSV-2.

DISCUSSION

Vaginal immunization with attenuated HSV-2 caused development of comparable protective immunity in both IgA KO and intact mouse strains, indicating that this immunity is not mainly caused by IgA. This observation agrees well with previous findings indicating that IgG and cell-mediated immunity are important mediators of this immunity.[11,20] Vaginal immunization with attenuated HSV-2 elicits relatively high ELISA titres of viral IgG antibody and low IgA titres.[11,13,21] The immune responses resulting from direct immunization of the vaginal mucosa with attenuated HSV-2 thus appear to differ from those arising in the gut or respiratory mucosa, where specific IgA antibody usually predominates.[2,3] This may result from a lack of organized immune inductive lymphoid tissue in the murine genital tract.[22] The IgG antibodies in vaginal secretions of immune mice are protective. This IgG neutralized challenge virus effectively whereas the IgA in vaginal secretions did not, and passive transfer of IgG from immune to non-immune mice provided significant protection against infection of the vaginal epithelium.[11] In the present study, the titres of specific IgG in vaginal secretions of intact and KO immune mice were similar, suggesting that IgG contributed similarly to immune protection in both groups. Also, the numbers of $CD4^+$ and $CD8^+$ T cells in the vagina were comparable in both intact and KO mice. The main difference between intact and KO immune mice was the replacement of IgA by specific IgM antibody in vaginal secretions of the KO mice. The extent to which IgM compensated for the lack of IgA in KO mice is unknown.

Although the evidence indicates that vaginal immunization

with attenuated HSV-2 elicits a high level of immunity that does not significantly involve S-IgA antibody, the latter might play a greater protective role if stronger S-IgA responses could be induced at this site. Thus, many investigators have studied immunization at IgA inductive sites, including the intestine, nasopharynx and pelvis, at least partly with a view towards the induction of S-IgA responses in the female genital tract (see cross-references in reference 10). Use of this approach to stimulate an IgA response against HSV-2 in the vagina has been limited. Gallichan & Rosenthal[23] showed that intranasal immunization of mice with adenovirus that expressed HSV-glycoprotein B stimulated the production of specific IgG and trace levels of IgA in vaginal secretions, but the animals showed only partial protection against neurological illness following vaginal challenge. The immunity of the nasally immunized mice was thus weaker than that of mice immunized vaginally with attenuated HSV-2, but the adenovirus vaccine may not replicate well in the mouse nasopharyngeal mucosa and, additionally, it expressed only one HSV glycoprotein. Also, Kuklin *et al.*[24] reported that intranasal immunization of mice with live HSV-1 stimulated IgA responses in vaginal secretions, prevented illness following vaginal challenge and reduced vaginal infection. However, only trace amounts of IgA were detected in vaginal secretions and the presence of IgG in vaginal secretions was not evaluated. Intranasal immunization with plasmid DNA encoding HSV-1 glycoprotein B resulted in little or no protection in the vagina after challenge.[24] Another approach to stimulate S-IgA antibody in the vagina is by vaginal boosting. Secondary immunization with HSV-2 appears to increase S-IgA antibody titres in vaginal secretions more than IgG antibody titres, but the relative neutralizing effectiveness of this IgA has not been determined.[16,21]

The results of the present study show similarities to reports on selective IgA deficiency in humans, which is caused by an arrest in the B-cell differentiation pathway.[25] It can be found in apparently healthy individuals as well as associated with a number of different disorders, including frequent mucosal infections.[25-29] The difference in susceptibility to infections in IgA-deficient individuals is not well understood, but mucosal production of IgM and/or IgG may compensate for the lack of IgA in some cases.[30-36]

In summary, our observations on IgA KO mice indicate that local immunization in the mouse vagina with attenuated HSV-2 elicits strong immune protection that is equivalent to that observed in intact mice. These data support previous findings that IgG anti-HSV-2 antibodies and T-cell-mediated immunity are the main effector mechanisms induced in the vagina by this form of immunization. Further studies of immune mechanisms elicited in the vagina by other routes of immunization and the relative immune protection afforded by such immunizations should assist in the development of vaccines to prevent sexual transmission of HSV-2 and other genital tract pathogens.

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