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Intranasal immunization of mice with herpes simplex virus type 2 recombinant gD2: the effect of adjuvants on mucosal and serum antibody responses

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SUMMARY

Mucosal immunization offers the potential for inducing IgA antibody responses in the vagina, the site of infection for many viruses, including herpes simplex type 2 (HSV-2). To investigate this possibility, mice were immunized intranasally with 10 μg glycoprotein D2 (gD2) from HSV combined with a series of adjuvants of proven efficacy; the oil in water emulsion MF59, poly(D,L-lactide-co-glycolide) microparticles (PLG) (encapsulated or co-administered), immune-stimulating complexes (iscoms) (incorporated or co-administered with iscomatrix) and the genetically detoxified enterotoxin from *Escherichia coli*, LT-K63. Encapsulation of gD2 into PLG microparticles, incorporation of gD2 into iscoms and co-administration of gD2 with LT-K63 induced mucosal IgA antibody responses (nasal wash, saliva and vaginal wash) which were greater than those induced by intramuscular administration of gD2 with MF59. Intranasal immunization with these formulations also induced substantial levels of serum IgG and neutralizing antibodies. These studies demonstrated that intranasal immunization with potent adjuvants is an effective means to induce mucosal antibody responses, even in the lower genital tract.

INTRODUCTION

The majority of infectious diseases are acquired initially through the interaction of pathogens with mucosal surfaces. Important mucosally acquired pathogens include influenza virus and *Bordetella pertussis*, which infect the respiratory tract, herpes simplex type 1, which is primarily transmitted orally, and herpes simplex virus type 2 (HSV-2) and human immunodeficiency virus (HIV), which typically infect the the genital mucosa. Genital infections with HSV-2 are prevalent world-wide[1] and HSV-2 is the major cause of genital ulceration in Europe and North America.[2] In these geographical areas, the seroprevalence in subjects 20–45 years of age is 10–20%[3] and there is an increasing incidence of neonatal HSV-2 infection.[4] Neutralizing antibodies to HSV-2 are predominantly directed towards the viral surface glycoproteins, especially glycoproteins D (gD) and B (gB).[5] Both gD and gB have been produced as C-terminal truncated recombinant proteins in Chinese hamster ovary cells.[6] Immunization with gD and gB in MF59 adjuvant induced humoral and cellular immune responses and protection from challenge in experimental animals.[7]

In previous studies, the emulsion adjuvant MF59 has been administered parenterally, by the intramuscular route. However, in the present studies, MF59 and additional adjuvants were administered mucosally, using the intranasal route. The predominant immunoglobulin found on mucosal surfaces is secretory IgA (sIgA), which has been shown to offer protection against mucosal challenge with infectious agents in small animal models.[8,9] The appearance of sIgA in the mucosal secretions is regulated and maintained through a common mucosal immune system (CMIS)[10] which links the different mucosal sites, e.g. the genital and respiratory tracts and the gut. Parenteral immunization is not thought to be an effective means by which to induce mucosal immunity, since sIgA is secreted by plasma cells located beneath the epithelium of the mucosal surfaces. These plasma cells are believed to be effectively stimulated only through mucosal immunization.[10]

In the current studies, we attempted to stimulate the induction of sIgA responses in the mucosal secretions, through intranasal immunization with gD2 and exploitation of the CMIS.

To promote the induction of potent immune responses following intranasal administration, we evaluated a number of adjuvants for mucosal immunization. The adjuvant, MF59, which is a squalene-in-oil emulsion,[11] was evaluated for mucosal immunization with gD2. Also evaluated were immune-stimulating complexes (iscoms),[12] micro-particles[13] and the genetically detoxified derivative of the heat-labile enterotoxin from *Escherichia coli* (LT-K63).[14] Each of these adjuvants has previously been shown to be effective for mucosal delivery of antigens. In the current studies, we evaluated the need for the antigen to be physically associated with the adjuvants wherever possible, i.e. with iscoms and microparticles. In addition, limited dose–response studies were performed to evaluate further the performance of some of the most promising adjuvants.

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MATERIALS AND METHODS

Animals

Female BALB/c mice, aged 6–8 weeks, were obtained from Charles River Laboratories (Wilmington, MA). Animals were rested for 1 week prior to immunization.

Antigen

The C-terminal truncated derivative of HSV-2 gD (gD2) was prepared by expression of C-terminal truncated derivative genes in Chinese hamster ovary (CHO) cells as previously described.[7] To prepare gD2 iscoms, a hydrophobically modified gD2 (hgD2) was synthesized by reaction with *N*-(4-maleimidylmethylcyclohexane-1-carboxyl)-1,2 dihexandecyl-sn-glycero-3-phosphoethanolamine) trimethylammonium salt (MMCC/DHPE) (Molecular Probes, INC. Eugene, OR). Glycoprotein D2 (0·5 mg/ml in 0·1 M sodium phosphate pH 8·0, 2% octyl glucoside) was reacted with 3·0 mg/ml MMCC/DHPE for 1 hr at 37°. The reaction was monitored by following the loss of unmodified gD2 by reverse phase high-pressure liquid chromatography using a Vydac 214TP59 C4 column (Vydac, Hesperia, CA). The hydrophobic product hgD2 was separated from excess reagent by ion-exchange chromatography on a TSK-gel heparin column (TosoHaas, Montgomery, PA). The antigenic integrity of the hgD2 was demonstrated by an enzyme-linked immunosorbent assay (ELISA), using a gD2-specific monoclonal antibody, D10G12 (Chiron Corporation), which recognizes a conformational epitope.

Adjuvants

The characteristics of the adjuvants used in these studies are shown in Table 1. MF59 emulsion was prepared as previously described.[11] Poly(lactide-co-glycolide) (PLG 50/50, 70 000–100 000 MW; Medisorb Technologies International, Cincinnati, OH) microparticles were prepared as previously described, using a solvent evaporation process.[15] The particle size distribution was determined using a Malvern Mastersizer (Malvern Instruments, Worcestershire, UK) The encapsulation efficiency of gD2 was measured with bicinichonic acid (BCA; Pierce Chemical Co., Rockford, IL), following alkaline hydrolysis of the microparticles as previously described.[16] The gD2–iscoms were a gift from Dr B. Morein (Swedish National Veterinary Institute, Uppsala, Sweden) and were prepared as previously described.[17] The incorporation of hgD2 into iscoms was determined by amino acid analysis. Iscomatrix was obtained from Iscotec (Uppsala, Sweden). LT-K63, a non-toxic mutant of *E.coli* heat-labile enterotoxin[14] was obtained from Dr M. Pizza (Chiron Biocine, Siena, Italy).

Vaccine formulations

All the formulations were prepared on the day of immunization as follows. MF59 + gD2 was prepared by mixing MF59 stock (42 mg/ml squalene in 0·01 M sodium citrate pH 6·5) 1 : 1 with gD2·(400 μg/ml in 10% glycine, 0·15 M NaCl, 0·01 M sodium citrate pH 6·5). PLG/gD2 (gD2 encapsulated at 0·5% w/w) was prepared by resuspending lyophilized microspheres into phosphate-buffered saline (PBS) at 40 mg/ml. PLG + gD2 was prepared by resuspending lyophilized PLG placebo microspheres into a solution of gD2 (200 μg/ml) in PBS at 2 mg/ml. Iscom/hgD2 was diluted to 200 μg/ml hgD2, 54 μg/ml detoxified QuilA (QHA + QHC; Iscotec, Uppsala, Sweden) in 0·15 M NaCl, 0·05 M sodium phosphate pH 7·0. Iscomatrix + gD2 was prepared by mixing iscomatrix (215 μg/ml QHA + QHC, 0·15 M NaCl, 0·05 M sodium phosphate pH 7·0) 1: with gD2·(270 μg/ml, 10% glycine, 0·15 M NaCl, 0·01 M sodium citrate pH 6·5). LT-K63 + gD2 formulations were prepared by dilution of LT-K63 (1·5 mg/ml, 0·15 M NaCl, 0·05 M sodium phosphate pH 7·0) with gD2 (1 mg/ml, 10% glycine, 0·15 M NaCl, 0·01 M sodium citrate pH 6·5) into PBS (0·15 M NaCl, 0·05 M sodium phosphate pH 7·0) to give 200 μg/ml gD2 and LT-K63 at the dose specified in each experiment (0·02–1 mg/ml).

Immunization protocol

All of the vaccine formulations described were evaluated following intranasal immunization. Fifty microlitres of each formulation were transferred to the nasal cavity of unanaesthetized mice by micropipette with Multi-Flex microcapillary tips (PGC Scientific, Frederick, MD); 50 μl of the MF59 + gD2 formulation was also administered by intramuscular injection (i.m.) to a single control group. All groups of mice (10 animals per group) were immunized three times, on days 0, 28 and 56.

Sample collection

Mucosal (nasal wash, saliva, vaginal wash and faecal extract) and serum samples were obtained 2 weeks after the third immunization, on day 72. Saliva and vaginal washes were

Table 1. Vaccine formulations

Formulation	Description	Particle size	Antigen association	Adjuvant molecule
PBS	Buffer control	NA	NA	no
MF59	Oil-in-water emulsion	250 nm	no	no
PLG/gD2	Polymer microsphere gD2 encapsulated	1 μm	yes	no
PLG + gD2	Polymer microsphere gD2 mixture	1 μm	no	
Iscom/gD2	Lipid/gD2 particle	40 nm	yes	Quil A
Iscomatrix + gD2	Lipid particle gD2 mixture	40 nm	no	Quil A
LTK-63 + gD2	*E. coli* enterotoxin, genetically modified	NA	no	LT-K63

collected using absorbent cellulose wicks as previously described[18] (Polyfiltronic Group, Inc., Rockland, MA). Saliva was collected by inserting the wick tip into the mouth for 1–3 min. Antibody was extracted from the wick into 375 µl of PBS and stored at −20°. Vaginal washes were obtained by insertion of 50 µl PBS into the vagina with a micropipette. After repeated flushing and aspiration, the vaginal lavage was absorbed by insertion of a single wick. Antibody was extracted into 400 µl PBS and stored at −20°. Nasal washes were collected from decapitated animals by back-flushing 0·5 ml PBS from the trachea. The nasal fluids were stored at −20°. Fecal pellets were collected over a 1-hr period. The pellets were pooled by group, weighed and extracted immediately by suspension and incubation in PBS (10% w/w, 30 min at room temperature) followed by centrifugation (12 000 *g*, 10 min at 4°). The supernatant was dialysed (50 000 MW cut-off) against PBS overnight at 4° and was stored at −20°. One hundred microlitres of whole blood was collected from each animal by orbital puncture. Clots were removed by centrifugation and the serum was stored at −20°.

Antibody assays

IgA (gD2-specific and total) and IgG (gD2-specific) were determined on pools of fluids obtained from groups of 10 animals each by ELISA using flash chemiluminescent (aequorin) detection.[19] To determine gD2-specific IgA, 50 µl of gD2 (5 µg/ml, PBS) was absorbed overnight at 4° to white Microlite-2 plates (Dynatech Laboratories, Chantilly, VA). The coated wells were blocked for 1 hr at 37° with 150 µl of 5% goat serum (Gibco BRL, Grand Island, NY) in assay buffer [25 mM Tris, 10 mM ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid (EGTA), 2 mg/ml bovine serum albumin (BSA), 0·15 M KCl, 0·5% Tween-20, pH 7·0; SeaLite Sciences, Inc., Bogart, GA]. The plates were washed three times with washing buffer [20 mM Tris, 5·0 mM ethylene diamine tetraacetic acid (EDTA), 0·15 M NaCl, 0·5% Tween-20, pH 7·0; SeaLite Sciences, Inc.], tapped and dried. Serum and mucosal fluids were pooled by group. Serum samples and a serum standard were initially diluted 1/1000 with blocking buffer. Nasal wash, saliva, vaginal wash and faecal extract were assayed directly. Three-fold serial dilutions of standard and samples were prepared on microtitre plates. Fifty microlitres were transferred into coated, blocked plates and incubated for 1 hr at 37°. The plates were then washed 6 times with washing buffer, tapped and dried. Fifty microlitres of biotinylated goat anti-mouse α-chain-specific antibody (EY-Laboratories, Inc., San Mateo, CA) diluted 1/1000 in blocking buffer was used as a first antibody. After 1 hr incubation at 37°, the plates were washed six times, tapped and dried. Subsequently, 50 µl of Streptavidin–Aqualite (SeaLite Sciences, Inc.) diluted 1/500 in washing buffer were added and the plates were incubated for 1 hr at 37°. Plates were washed six times, tapped, dried and placed in a Dynatech ML-3000 Luminometer (Dynatech Laboratories). The luminescence was triggered by sequential injections of 50-µl aliquots of buffered calcium acetate (SeaLite Science, Inc.) to each well, followed by a 3-second determination of total luminescence. Primary data are reported as relative light units. Total IgA was determined in the same manner using plates coated with goat anti-mouse α-chain antibody (5 µg/ml, PBS) (Cappel Research Products, Durham, NC). IgG specific for gD2 was determined as above, except that biotinylated goat anti-mouse IgG (Sigma, St. Louis, MI) diluted 1/1000 was used as a first antibody. End-point titres were determined by extrapolation of plots of logarithm of luminescence versus logarithm of dilution to two standard deviations above background.[19] Standard deviations were determined for independent assays on single mucosal extracts and significance of data was determined using the two-tailed *t*-test as calculated by Microsoft Excel 5·0.

Virus neutralization assay

HSV-2 neutralizing titres were determined as previously described,[20] using a plaque reduction assay with a twofold serial dilution. The titre reported is the reciprocal of the serum dilution required to inhibit the cytolysis of a confluent monolayer of Vero cells by 50%.

RESULTS

The series of adjuvants described in Table 1 were each evaluated intranasally at a dose of 10 µg of gD2. The MF59 adjuvant was also used at a 10-µg dose of gD2 for i.m. immunization. Titres of gD2-specific IgA were determined for mucosal fluids (nasal wash, saliva, vaginal wash and faecal extract) as described in the Materials and Methods. For all the mucosal samples, IgA titres were corrected for dilution factor during processing to give the titre in the primary sample. However, no attempt was made to calculate the dilution factor from mucosal samples collected by washes. It is therefore not productive to make comparisons of the titres obtained with any adjuvant at various mucosal sites based upon this data set. In all assays, IgA titres for the groups were determined from pooled samples. Figure 1 shows the IgA responses obtained in mucosal samples collected 2 weeks after the third immunization. Samples obtained from nasal wash following intranasal immunization with three of the formulations, gD2 in microparticles (PLG/gD2), gD2 in iscoms (iscom/gD2) or gD2 administered with LT-K63 (gD2+LT-K63), showed significantly higher IgA antibody responses than the responses obtained by intranasal immunization in the absence of adjuvant ($P<0·05$). The formulations could be ranked according to IgA titre in the order gd2/PLG > iscom/gD2 > gd2+LT-K63 ($P<0·05$). None of the remaining formulations (MF59+gD2, PLG+gD2 and Iscomatrix+gD2) produced IgA titres which were significantly greater than that achieved with gD2 alone. Determination of IgA in saliva showed a similar pattern with PLG/gD2 and iscom/gD2 producing higher titres than those achieved with gD2 alone ($P<0·05$). IgA titres achieved with LT-K63 were not significantly different from those obtained with gD2 alone ($P<0·05$), however, the value was not distinguishable at $P<0·05$ from that achieved with the iscom formulation. The results obtained at a more distal site from vaginal wash are similar to those obtained from saliva and nasal wash. Iscom/gD2 and gD2+LT-K63 groups have titres significantly greater than those found with gD2 alone ($P<0·05$). Titres for the PLG/gD2 group fail by a small margin at $P<0·05$ due to poor precision between replicates which renders the group not significantly different from the Iscom or LT-K63 groups as well. IgA titres obtained from faecal wash after intranasal immunization rank LT-K63+gD2 > PLG/gD2 ($P<0·05$) > gD2 alone ($P<0·05$). For nasal wash, saliva and vaginal wash IgA titres obtained by intranasal immunization are significantly

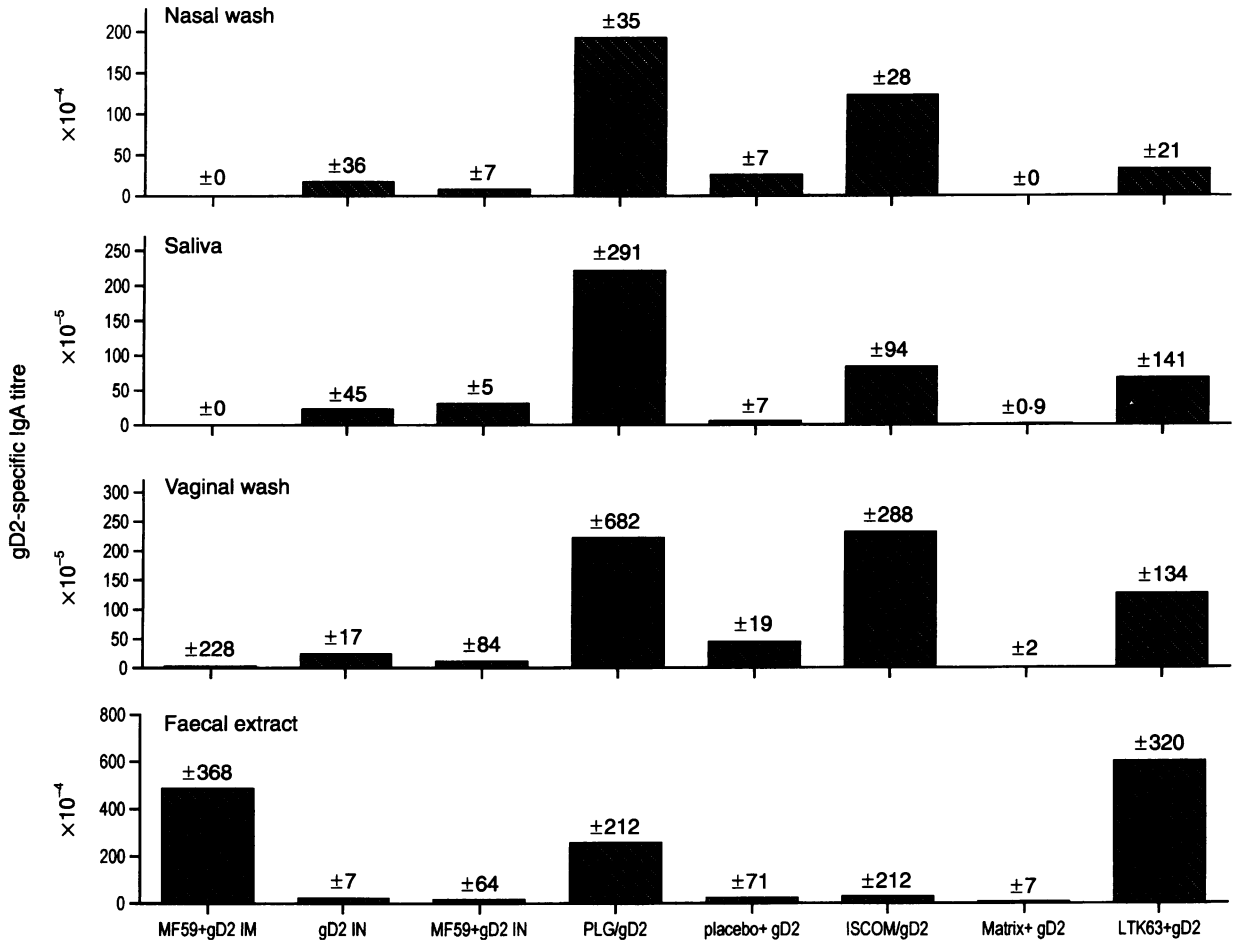

Figure 1. HSV gD2-specific IgA titres: mean titres obtained from replicate ELISA analyses on pooled mucosal samples (nasal wash, saliva, vaginal wash and faecal extract) obtained 2 weeks after the third immunization. All groups received 10-μg doses of gD2 antigen formulated with the following adjuvant doses: MF59 (1·0 mg squalene), PLG (2·0 mg), iscom/Iscomatrix (1·2 μg Quil A), or LT-K63 (50 μg).

greater than those achieved by i.m. imunization with MF59 + gD2. In the case of faecal wash however, titres obtained from i.m. immunization are significantly greater than those obtained with gD2 alone ($P < 0{\cdot}05$) and not significantly different from those obtained with LT-K63, the most potent intranasal adjuvant. These data suggest the possibility that transudation from serum may be an important mechanism for generation of faecal antibody. Across the full data set the striking observation can be made that the antigen must be incorporated into PLG microparticles, or associated with iscoms for an adjuvant effect to be achieved. Antigen mixed with PLG or Iscomatrix was not effective as an intranasal adjuvant.

Data from Fig. 1 allow a comparison of adjuvant strength in various mucosal fluids, but cannot provide an estimate of the relative antibody titres obtained at the different mucosal sites. In order to allow this comparison of IgA titres at various mucosal sites, we repeated the study and determined both gD2-specific IgA and total IgA titres for the mucosal samples.

Figure 2 shows the ratio of gD2-specific IgA to total IgA obtained in the four mucosal samples. The activity ratios for a given formulation are remarkably similar for nasal wash, saliva and vaginal wash. For example the PLG/gD2 formulation produced gD2-specific IgA : total IgA ratios ranging from 2 to 5 across the series of samples. The results for faecal extract are very different. The highest ratios observed are a thousand times lower than those found in the remaining samples, with a maximum ratio of 4×10^{-3} for gD2 + LT-K63. These results also appear to indicate that the factors controlling IgA presence in the gut are very different from those in the remaining mucosal samples tested. The ratio data also provide an additional opportunity to rank adjuvant formulations for each sampling site. The ratio data provide a similar picture to that obtained from the gD2-specific IgA titres. For nasal wash IgA, the previously significant formulations can be ranked PLG/gD2 > iscom/gD2 > gD2 + LT-K63 ($P < 0{\cdot}05$), and for saliva PLG/gD2 = iscom/gD2 > gD2 + LT-K63 ($P < 0{\cdot}05$). For vaginal wash the apparent rankings remain the same however, the data are not significant at $P < 0{\cdot}05$. Ranking of adjuvants for IgA titre in the faecal extract is similar to the pattern seen previously: LT-K63 (intranasal) = PLG/gD2 (intranasal) = gD2 + MF59 (i.m.) > gD2 (intranasal) ($P < 0{\cdot}05$). These data reinforce the previous conclusions that three adjuvants show substantial potency. PLG/gD2 and iscom/gD2 are particularly potent in nasal wash, saliva and vaginal wash where i.m. gD2 + MF59 is ineffective.

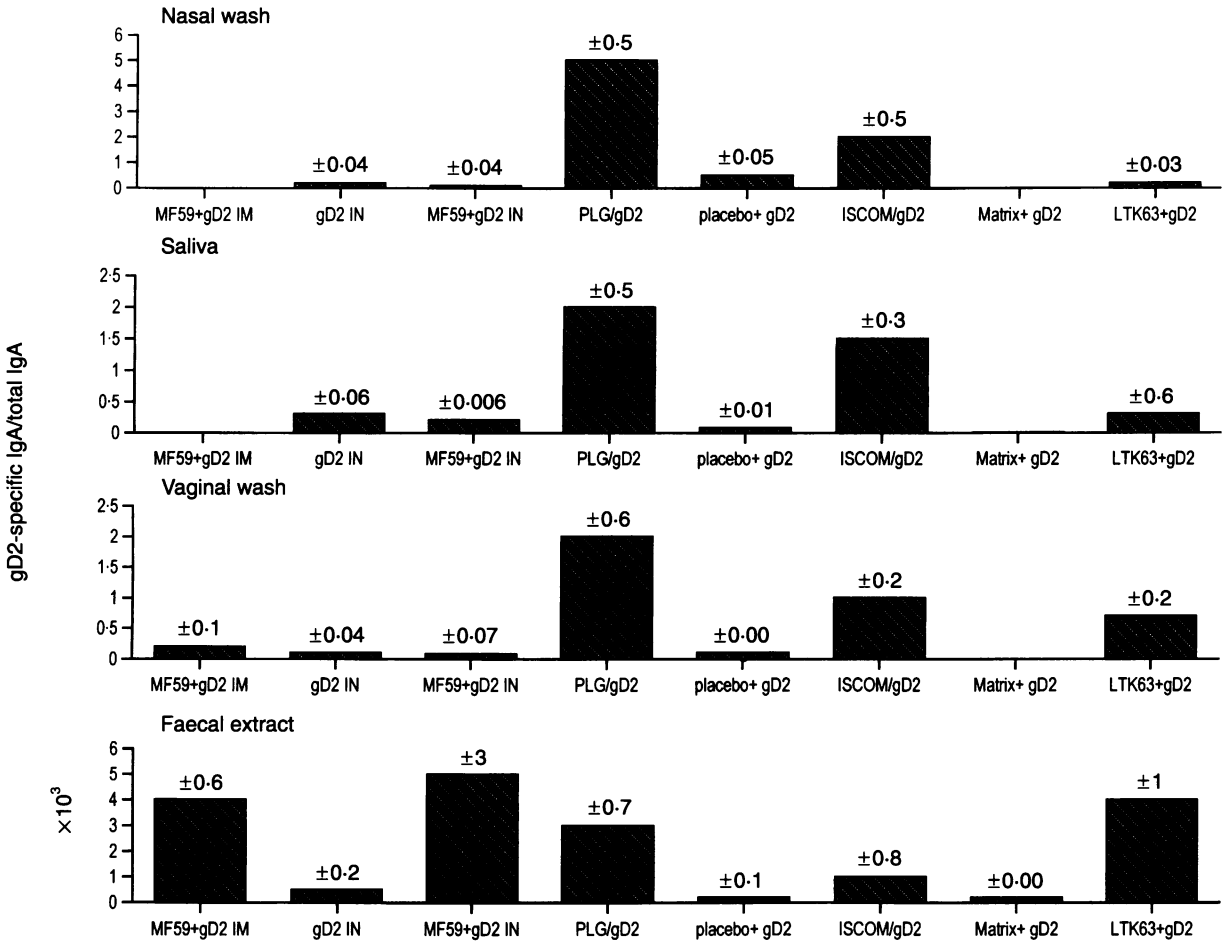

Figure 2. Ratios of HSV gD2-specific titre: total IgA titre: ratios of mean gD2-specific IgA titre to mean total IgA titre obtained from replicate ELISA analyses on pooled mucosal samples (nasal wash, saliva, vaginal wash and faecal extract) obtained 2 weeks after the third immunization. All groups received 10-μg doses of gD2 antigen formulated with the following adjuvant doses: MF59 (1·0 mg squalene), PLG (2·0 mg), Iscom/Iscomatrix (1·2 μg Quil A), or LT-K63 (50 μg).

LT-K63 and PLG/gD2 are of similar potency to gD2 + MF59 i.m. in the faecal extract.

Doses of both antigen and adjuvant expected to generate high-level responses were chosen for the experiments shown in Figs. 1 and 2 in order to compare the different adjuvants. Dose–response curves were evaluated for the two most promising adjuvants (Fig. 3). A single microparticle composition (PLG 50/50, 0·5% gD2) was intranasally administered at 1, 10 and 100 μg of gD2 and the IgA responses were determined in mucosal samples in the fashion previously described. For nasal wash, saliva and vaginal wash, the IgA responses induced with 1 μg gD2 were significantly reduced from those achieved with 10 μg, $P<0·01$ (Fig. 3). Increasing the dose to 100 μg gD2 resulted in a substantial increase in gD2-specific IgA only for saliva. No dose–response could be discerned for the faecal extracts. On the basis of these mixed data we conclude that 10 μg of gD2 in the current microparticle formulation appears to be a suitable working dose for intranasal administration in mice. For the LT-K63 + gD2 formulation, in which the antigen and adjuvant dose are independent variables, the antigen dose was maintained at 10 μg, while the LT-K63 dose was varied from 0 to 50 μg (Fig. 3b). Data obtained from all of the mucosal fluids show that a 1-μg dose of LT-K63 gives rise to a significant increase in IgA over that achieved with antigen alone ($P<0·05$). This is in contrast to immunization in the presence of 1 μg cholera toxin B subunit (CTB, non-recombinant with undetermined CT contamination) where IgA titres were indistinguishable from those generated with gD2 alone (data not shown). Data from the nasal wash, saliva and vaginal wash samples do not show a clear trend in dose–response. The apparent increase between 25 μg and 50 μg for saliva and vaginal wash are not significant at $P<0·05$. For the faecal extract sample a significant increase ($P<0·05$) is observed between 25 μg and 50 μg. This observation reinforces the idea that the effects of intranasal immunization on faecal IgA are different from those observed for the three other samples.

Although the primary objective of these studies was to determine the levels of IgA antibodies induced by intranasal immunization, transudated IgG may be the protective agent in the mouse HSV model. IgG specific for gD2 and HSV-2 neutralizing antibodies in serum were determined for the full series of vaccine formulations. While intramuscular immunization with gD2 + MF59 results in a virus-neutralizing titre an order of magnitude greater than those obtained by the intranasal route, intranasal immunization with gD2 formulated

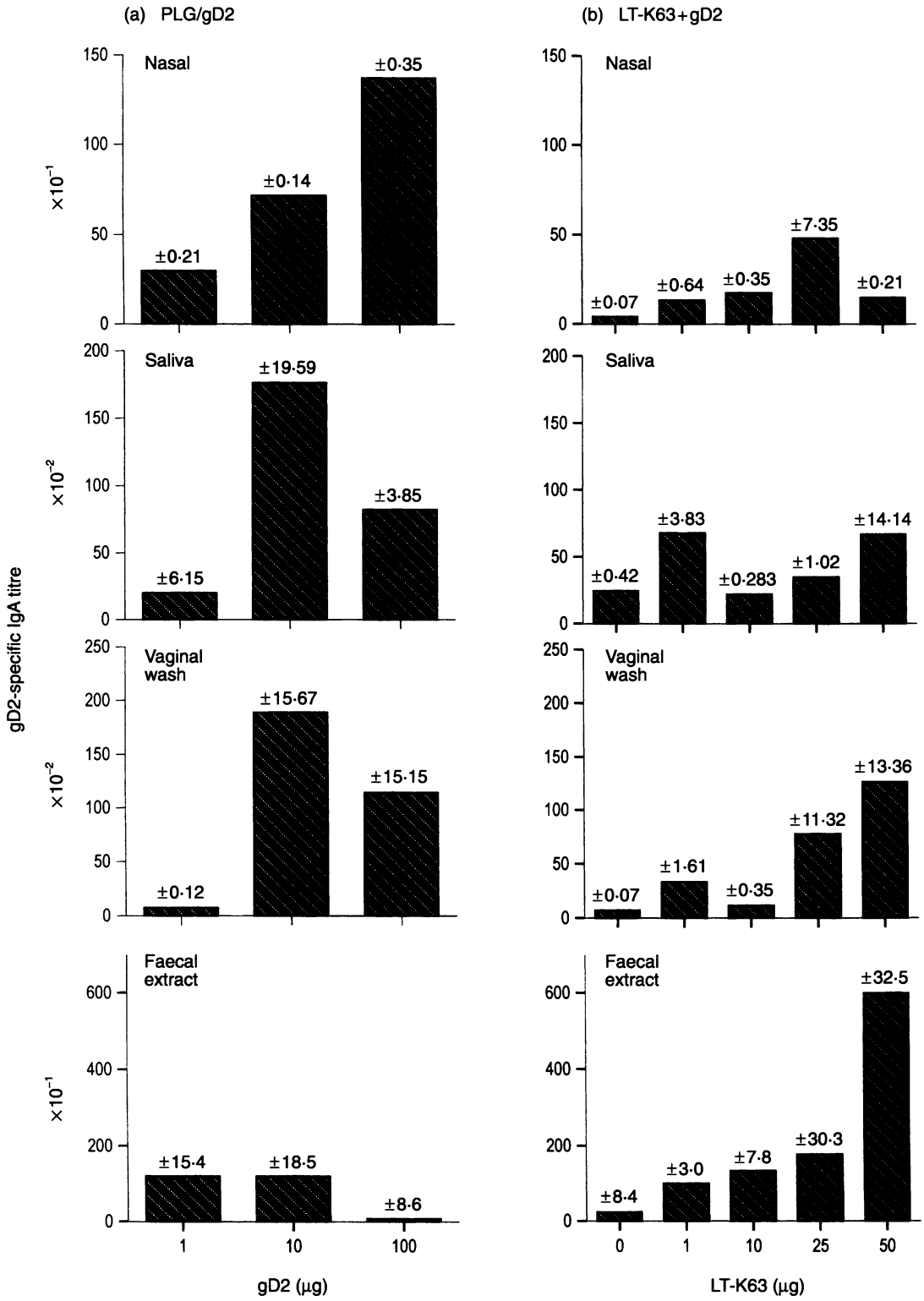

Figure 3. HSV gD2-specific IgA titres from primary mucosal samples (nasal wash, saliva, vaginal wash and faecal wash) obtained two weeks after the third immunization. (a) Response as a function of gD2 dose (PLG/gD2 microspheres); (b) response as a function of LT-K63 dose at a constant gD2 dose of 10 μg.

with LT-K63, PLG microspheres, MF59 and in iscoms does result in substantial neutralizing activity in the serum (Fig. 4). Intranasal immunization with 50 μg LT-K63+gD2 produced an IgG titre greater than that found with MF59+gD2 i.m. ($P<0·05$). The intranasal formulations were ranked by IgG titre: LT-K63>PLG/gD2, iscom/gD2>gD2 ($P<0·05$). These data demonstrate that intranasal immunization with HSV-2 gD2 and an appropriate adjuvant can provide significant

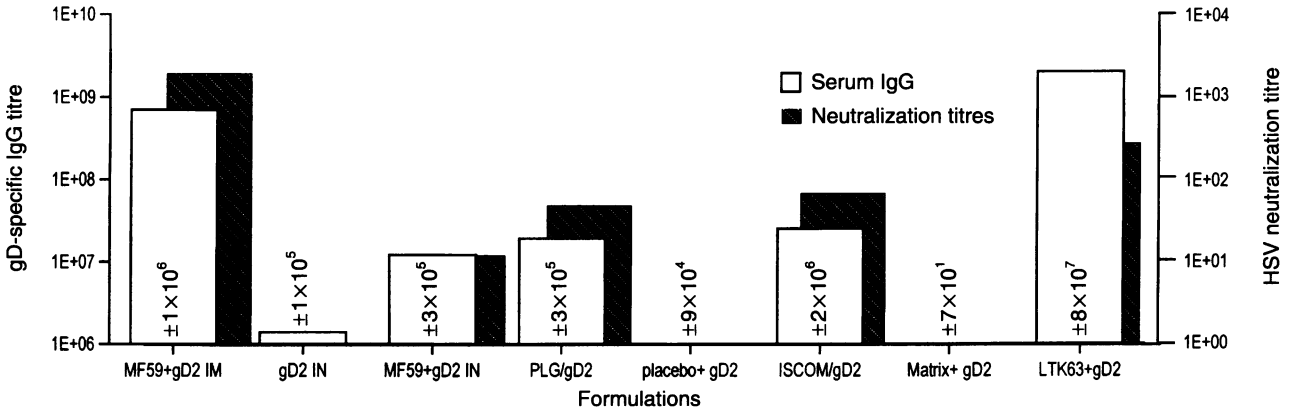

Figure 4. Log_{10} serum titres obtained 2 weeks after the third immunization. (a) Log_{10} gD2-specific IgG titre; (b) Log_{10} HSV-neutralizing titre. All groups received 10-μg doses of gD2 antigen formulated with the following adjuvant doses: MF59 (1·0 mg squalene), PLG (2·0 mg), iscom/Iscomatrix (1·2 μg Quil A), LT-K63 (50 μg).

systemic response in addition to the anticipated improved mucosal effects.

DISCUSSION

The results obtained in the current studies served to underline the significant potential of LT-K63, PLG microparticles and iscoms as mucosal adjuvants for intranasal immunization. All three of these adjuvants induced significantly better mucosal IgA responses in mucosal samples than intranasal immunization with gD2 alone, or intranasal immunization with gD2 combined with either the emulsion adjuvant, MF59 or a 1-μg dose of CTB. These results are compatible with those of Giuliani *et al.*[21] who found that titres obtained by intranasal immunization of mice with ovalbumin were significantly enhanced by addition of 1 μg LT-K63, but not LTB and showed that 1 μg wild-type LT was approximately ten times more effective than 1 μg LT-K63. In addition, these same adjuvants generally induced significantly better mucosal IgA responses than i.m. immunization with gD2 in MF59 or oral immunization with PLG/gD2 (data not shown). These results confirmed that intranasal immunization in mice may be successfully exploited to induce disseminated mucosal immune responses at a number of sites, including the lower genital tract. The induction of vaginal wash IgA responses is particularly interesting, since the current pathogen under investigation, HSV-2, infects the lower genital tract.

The specific activity of IgA was greater in the secretions proximal to the site of immunization, the nasal cavity. Lower values for specific activity of IgA were obtained for the more distal mucosal sites, the vaginal wash and faecal extracts. Previous studies in rodents have demonstrated that the CMIS is subject to compartmentalization and that mucosal immunization generally stimulates optimal IgA responses at the site of antigen delivery.[18] Nevertheless, intranasal immunization in rodents[22] and primates[23] has been shown to induce disseminated mucosal IgA antibody responses.

LT-K63 is a potent adjuvant and induced the highest levels of serum neutralizing antibodies following intranasal administration. Although LT-K63 was used at a higher dose than used previously[24] the dose–response studies confirmed that LT-K63 is an effective adjuvant at a dose of only 1 μg. However, higher doses showed greater efficacy for inducing a disseminated mucosal response. We do not currently know the upper dose for optimal efficacy of LT-K63 in the mouse model, nor do we know the optimal dose for efficacy or safety in humans by this route. In a clinical trial involving intranasal administration of an influenza vaccine, LTB at a dose of 99·5 μg (with 0·5 μg of native LT) was shown to be safe and effective in humans.[25] In previous studies, LT-K63 has been shown to be at least 1000-fold less toxic than native LT.[14]

In previous studies involving intranasal immunization with bacterially derived proteins[26,27] or a chemically toxoided antigen[28] microparticles were shown to induce protective immunity in mice against aerosol challenge. The current studies demonstrated that microparticles are also an effective mucosal adjuvant for intranasal immunization with an entrapped recombinant protein. The induction of neutralizing antibodies following immunization with microparticles is important, since it has been reported that microencapsulation can result in denaturation of entrapped antigens.[29] The dose–response studies with microparticles demonstrated that 10 μg of gD2 was the optimal dose for this particular microparticle formulation in mice. However, better responses may be obtained with alternative microparticle formulations in which the loading level of the entrapped protein is varied. In a previous study, it was shown that the antigen load and the total number of particles administered affected the immunogenicity of microparticles.[30] Nevertheless, the current studies were only concerned with determining the dose–response relationship for the single microparticle formulation under evaluation. These studies also demonstrated that intranasal immunization with iscoms is an effective means by which to induce mucosal IgA responses. Iscoms formulated from palmitified p27 (recombinant *Eimeria falciformis* sporozoite coat protein) and palmitified ovalbumin have previously been shown to be effective for mucosal immunization.[31,32] The induction of vaginal IgA responses following intranasal immunization is a particularly encouraging observation, since intravaginal immunization has previously been shown to be a poor means by which to induce local IgA responses.[33,34]

In the current studies, it was clear that the antigen needed to be physically associated with the microparticles, or the iscoms, to exert an adjuvant effect. This is probably a

consequence of the ability of the particulate carrier systems to deliver associated antigens into the nasal associated lymphoid tissue (NALT). The need for antigens to be physically associated with microparticles for an adjuvant effect has previously been evaluated for the parenteral route. Eldridge *et al.*[35] reported that microparticles were not an effective adjuvant for unentrapped antigen. However, O'Hagan *et al.*[36] showed that microparticles were an effective adjuvant for an adsorbed protein, an observation consistent with historical data.[37] It has similarly been reported that the adjuvant effect of iscoms is greater if the antigen is physically associated with the iscom.[38] In the current intranasal studies, no adjuvant effect was observed when antigen was simply mixed with Iscomatrix. Some caution should be exercised in interpreting the data entirely on the basis of antigen association since minor compositional differences between the iscoms utilized and Iscomatrix may be relevant. MF59 has previously been shown to be an effective adjuvant for parenteral immunization with antigens that show no physical association[11] but was not an effective adjuvant for gD2 intranasally.

Microparticles appeared to be the most effective adjuvant for the induction of specific IgA responses. However, LT-K63 was the most effective intranasal adjuvant for the induction of serum neutralizing antibodies. This might indicate that these two adjuvants work through distinctly different mechanisms.

In summary, PLG microparticles, iscoms and LT-K63 were shown to be effective intranasal adjuvants for the recombinant protein gD2. These adjuvants induced disseminated mucosal IgA antibody responses, serum antibodies and neutralizing antibodies. The mucosal IgA responses induced were significantly greater than the responses induced by i.m. immunization with a potent systemic adjuvant. A striking observation was the apparent need for physical association between the particulate carriers, iscoms and microparticles, and the antigen for the adjuvant effect following intranasal immunization.

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