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Initial Route of Antigen Administration Alters the T-Cell Cytokine Profile Produced in Response to the Mouse Pneumonitis Biovar of *Chlamydia trachomatis* following Genital Infection

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A Th1-type response develops following vaginal infection with the mouse pneumonitis biovar of *Chlamydia trachomatis* (MoPn). Since the type of response, i.e., Th1 versus Th2, can be influenced by factors present during T-cell activation, we examined the effects of different routes of MoPn administration on the cytokine profile and resistance against infection following a MoPn vaginal challenge. A dominant Th1-type cytokine profile developed in mice given live MoPn via the intranasal, oral, and vaginal routes with ratios of gamma interferon-secreting cells to interleukin 4-secreting cells greater than 10. In contrast, mice injected subcutaneously produced a Th2-type profile with a gamma interferon/interleukin 4 ratio of only 0.7. These mice also had significantly higher anti-MoPn immunoglobulin G1 serum titers, confirming a Th2-type cytokine profile. Exposure of mice to live MoPn, by any route prior to vaginal challenge, resulted in a shortened course of infection. However, the subcutaneous group resolved the vaginal infection more slowly, with 60% (6 of 10 mice) of the mice still isolation positive 12 days after challenge compared with only 20% of mice given live MoPn by other routes. Administration of UV-inactivated MoPn did not provide protection against a vaginal challenge. The decreased ability to clear infection was not associated with a shift in the cytokine profile, since intranasal and oral administration of UV-inactivated MoPn resulted in a predominant Th1-type response. Taken together, these data indicate that the initial route of MoPn administration can direct the type of response produced after a local MoPn infection and thus influence the ability of the immune response to protect against subsequent infection.

Chlamydia trachomatis genital infection in women is a significant cause of pelvic inflammatory disease and can cause infertility. While *C. trachomatis* infections are treatable with antibiotics, most are subclinical infections and are not detected until after significant damage has occurred (6). A successful strategy for preventing pathologic damage due to *C. trachomatis* infection would be to reduce the severity and length of infection by priming individuals through vaccination. While immunization with the appropriate antigen(s) is necessary to elicit protection, it is becoming increasingly clear that immune deviation (2) can significantly influence the outcome of an immune response to infection by altering the production of T-cell cytokines and immunoglobulin isotypes. The regulation of immune deviation can occur by a variety of factors such as the dose of antigen (7), the adjuvant used (3) and even the site of injection (23). The type of immune response (Th1 or Th2) can result in protective, nonprotective, and even immunopathologic responses to chlamydiae (14, 29) as well as other organisms (33). Understanding the role these factors play in shaping the immune response to *C. trachomatis* is essential for developing future vaccine regimens.

An immune response to *C. trachomatis* is produced following natural infection in humans with production of both cell-mediated responses and specific antibody to chlamydiae. This immunity is short-lived but can provide some protection (15, 32). Characterization of the type of immune response necessary for resolution of *C. trachomatis* genital infection has been actively investigated for a number of years in animal models (26). While species differ somewhat in the types of immune responses necessary for resolution and protection (cell mediated versus antibody), these studies all highlighted $CD4^+$ T cells as the essential subset (21, 27, 36). Recent studies with the murine model of *C. trachomatis* genital infection have further defined the $CD4^+$ T-cell response as a Th1 type with relatively high levels of gamma interferon (IFN-γ) and low levels of interleukin 4 (IL-4) (4, 12).

The choice between a Th1 response and a Th2 response occurs early during T-cell activation and is influenced by factors present in the local microenvironment, such as the relative levels of IFN-γ and IL-4 (19, 34). Other factors within the local microenvironment that are responsible for shaping $CD4^+$ T-cell subset development include the expression of certain costimulatory molecules on antigen-presenting cells (16) and production of other cytokines, such as IL-12, from neighboring cells (39). Once a polarized Th1 or Th2 cell population has developed, the cytokine pattern is relatively stable and resistant to regulatory influence (1, 37). Therefore, the local microenvironment in which an immune response is initiated appears to shape and "imprint" that particular type of immune response, so that upon a subsequent exposure to the same infection, an identical type of immune response is produced. With respect to vaccine development, the route of immunization or site of administration may play a prominent role in shaping the type of immune response that develops. In support of this concept, a recent report has shown that the route of immunization influenced the ability of chickens to prevent lung infections following intratracheal challenge with *Mycoplasma gallisepticum* (24). Here, we report experiments examining the

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influence of the site of administration of the mouse pneumonitis biovar of *C. trachomatis* (MoPn) on the cytokine pattern (Th1 versus Th2) which develops in response to a vaginal challenge with MoPn and the ability of different routes to protect mice from developing a vaginal infection.

MATERIALS AND METHODS

Animals. Female BALB/c mice, 4 to 6 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.) and were given food and water ad libitum. Mice were allowed to acclimate to the American Association for Accreditation of Laboratory Animal Care-accredited housing environment for approximately 1 week before beginning experimentation.

MoPn administration. Mice were given live MoPn (American Type Culture Collection, Rockville, Md.) grown in HeLa or McCoy cells or UV-inactivated MoPn antigen that was purified over a Renografin gradient (5) via intranasal, subcutaneous, oral, and vaginal routes. For the intranasal, oral, and vaginal administration groups, MoPn was administered while mice were anesthetized with sodium pentobarbital anesthesia. Intranasal administration was performed by allowing the inhalation from the nares of MoPn in sucrose phosphate-glutamate buffer in two 25-μl applications. MoPn was delivered orally through a feeding needle (Popper and Sons, New Hyde Park, N.Y.) in 100 μl in sucrose phosphate-glutamate buffer followed by a 50-μl rinse with phosphate-buffered saline (PBS). Mice were monitored following each feeding for aspiration through the nares. Subcutaneous administration was performed by injecting MoPn between the shoulder blades. Vaginal administration was performed by depositing 1×10^7 inclusion-forming units (IFU) of live MoPn in 30 μl of sucrose phosphate-glutamate buffer in the vaginal vault while under sodium pentobarbital anesthesia. To ensure infection in cycling mice, three consecutive doses of live MoPn (1×10^7 IFU) were given. This regimen was extensively used in the past to reliably induce genital infection (4) and served as our positive control. Doses of live MoPn had to be adjusted either to prevent significant mortality (intranasal) or morbidity (oral) or to enhance the ability of MoPn to infect nonmucosal sites (subcutaneous). The following doses were used: intranasal, 1×10^3 IFU; oral, 1×10^5 IFU; and subcutaneous, 1×10^8 IFU. The dose of UV-inactivated MoPn was equivalent for all routes (50 μg), with the exception of the intranasal route. In order to compare the intranasal administrations of live and UV-inactivated MoPn, the dose was reduced to 25 μg of UV-inactivated MoPn antigen to compensate for the greatly reduced dose of live MoPn.

Infection. Two weeks after the second dose, all groups were injected subcutaneously with 2.5 mg of DEPO-PROVERA (Upjohn, Kalamazoo, Mich.) in 100 μl of sterile PBS. DEPO-PROVERA drives mice into a state of anestrus, thus eliminating the variability in the rate and severity of infection due to the estrous cycle (28). Seven days later, while under sodium pentobarbital anesthesia, all mice were inoculated with 10^7 IFU of MoPn grown in McCoy cells. Infection was monitored every 3 days after infection with cervico-vaginal swabs (Dacroswab Type 1; Spectrum Laboratories, Houston, Tex.) in five mice from each group. The swabs were stored at −70°C in sucrose-phosphate buffer (31) until analyzed.

Isolation of chlamydiae from cervico-vaginal swabs. Swabs were vortexed for 1 min in the presence of glass beads to release chlamydial organisms from the swab. Eagle's minimal essential medium containing 10% fetal bovine serum, 5% glucose, 100 μg of vancomycin per ml, 50 μg of gentamicin per ml, 2.5 μg of amphotericin B (Fungizone) per ml, and 0.5 μg of cycloheximide per ml was added to each tube, for a final volume of 2.3 ml. Individual wells of McCoy cell monolayers in 96-well plates were inoculated with 200 μl of the solution described above followed by centrifugation at 1,900 × *g* for 1 h. The plates were then incubated for 2 h at 37°C. At this time, the isolation solutions were removed, fresh medium was added, and the plates were incubated for an additional 32 h. The cultures were then fixed with methanol. MoPn inclusions were identified by the addition of anti-MoPn immune sera and anti-mouse immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (ICN Immunobiologicals, Irvine, Calif.). The number of inclusion bodies within 20 fields (40×) was counted under a fluorescent microscope, and inclusion-forming units per milliliter were calculated. Mice were considered free from infection when no inclusion bodies were detected at two consecutive time points.

Anti-MoPn enzyme-linked immunosorbent assay (ELISA). Sera from mice were collected at various times by retroorbital bleeds and stored at −20°C until analyzed. Microtiter plates were coated with 5-μg/ml UV-inactivated MoPn antigen grown in HeLa cells and purified over Renografin gradient in 0.5 M $NaHCO_3$ overnight at room temperature, and then blocked with a 5% solution of horse serum in PBS for 30 min. Serum samples were applied to the plate in serial dilutions, incubated at 37°C for 1 h, and washed with PBS-Tween 20. Goat anti-mouse IgG1 (1:5,000 dilution) or IgG2a (1:10,000 dilution) conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, Ala.) was then applied to all wells, incubated at 37°C for 1 h, and then washed. The bound antibody was then visualized by adding the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (Sigma, St. Louis, Mo.) in 0.1 M citrate buffer, pH 4.35, at a concentration of 0.3 mg/ml. Optical densities were read after 1 h at 405 nm. The titer for individual mice was determined as the last dilution with an optical density value greater than that of the control wells.

Isolation of lymphoid cells. Single-cell suspensions of spleen, iliac lymph nodes (ILN), and mesenteric lymph nodes (MLN) were pooled from 10 mice per group in RPMI 1640 (Gibco, Gaithersburg, Md.), minced with scissors, and expressed through a 70-μm-pore-size nylon mesh. Erythrocytes were lysed with 0.17 M Tris-NH_4Cl, and the cells were then washed twice and suspended in complete RPMI 1640 medium containing 10% fetal bovine serum, 100 U of penicillin per ml, 100 μl of streptomycin per ml, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10 mM (each) nonessential amino acids, sodium pyruvate, and HEPES.

ELISPOT assay. We determined the frequency of IFN-γ- and IL-4-secreting cells with the enzyme-linked immunospot (ELISPOT) assay (38). Wells of nitrocellulose-based, 96-well plates (Millititer HA; Millipore Corporation, Bedford, Mass.) were coated with 2 μg of antibodies per ml directed against murine IFN-γ or IL-4 (PharMingen, San Diego, Calif.) overnight at 4°C. Prior to the addition of cells, the plates were blocked with medium containing 5% fetal bovine serum for 1 h at 37°C. Cells (1×10^7/ml) were first incubated with 5-μg/ml UV-inactivated MoPn antigen grown in HeLa cells and purified over Renografin gradient (5) or 5-μg/ml concanavalin A (Sigma) in complete RPMI medium overnight in 24-well microtiter plates (Costar, Cambridge, Mass.) before being applied to the coated wells. Dilutions ranging from 10^3 to 10^5 cells were then added to individual wells and incubated overnight at 37°C in 5% CO_2. The plates were then washed twice with PBS containing 0.1% Tween 20; this was followed by an overnight incubation at 4°C with 4 μg of a different biotinylated antibody against IFN-γ or IL-4 per ml. The plates were then washed and incubated for 1 h with 2.5 μg of avidin-peroxidase (Vector, Burlingame, Calif.) per ml, and cytokine bound to specific antibody was visualized with 3-amino-9-ethylcarbazole (Vector). No spots were seen in the control wells that were not coated with anticytokine antibodies. The mean number of cytokine-secreting cells per 10^6 cells was determined from triplicate wells.

Proliferation assay. Single-cell lymphoid populations (2×10^5) were incubated with MoPn or concanavalin A as described above for 5 days in flat-bottom, 96-well microtiter plates (Costar). Proliferation was measured by [^{3}H]thymidine incorporation (1 μCi) over the last 18 to 24 h of the incubation period. Cells were harvested onto a Self-Aligning RG glass fiber filter (Packard, Meriden, Conn.) with a Packard FilterMate cell harvester. Determination of total counts over an 8-min period was obtained by a Packard Matrix 96 Direct Beta Counter. The mean of triplicate wells was determined and used to calculate the stimulation index (mean proliferative response to antigen/mean proliferative response of control wells in the absence of antigen).

Statistics. The mean numbers of inclusion-forming units per milliliter, cytokine-secreting cells per milliliter, and antibody titers ($\log_{10}$ transformation) for all groups were compared at each time point by using the Kruskal-Wallis, one-way analysis of variance (ANOVA) and *t* test with Bonferroni's correction. Statistical differences in the incidence of recovery from MoPn vaginal challenge were determined with Fisher's exact test.

Experimental design. Groups of 15 mice were given live or UV-inactivated MoPn as described above. All of the groups, except the group receiving live MoPn intranasally, were given a second inoculation 2 weeks after the first dose. Sera were collected for anti-MoPn antibody levels 2 to 3 weeks after the second dose. At week 5 after the initial exposure, all mice were inoculated intravaginally with live MoPn as described above. One week later, lymphoid tissues were removed from 10 mice in each group for ELISPOT and proliferation assays. The remaining mice were monitored for MoPn shedding in the genital tract with cervico-vaginal swabs. The numbers of mice required for each experimental group prohibited experiments including all groups; therefore, each experiment contained one route including both live and UV-inactivated MoPn groups and the vaginal control group.

RESULTS

Comparison of the cytokine profiles in response to live MoPn given via different routes. The cytokine profile of T cells in response to specific antigens comprises cytokines from both Th1- and Th2-type responses. Designation of one type of response versus the other depends on which pattern dominates. The cytokine profile of T cells in response to MoPn antigen following vaginal challenge in BALB/c mice reflects a Th1-type profile with high levels of IFN-γ and low levels of IL-4 (4). To examine whether administration of MoPn at different anatomical sites influences the nature of the immune response to MoPn in the genital tract, we compared the ratio of IFN-γ- to IL-4-secreting cells in ILN, which drain the genital tract, 7 days after a vaginal challenge with MoPn. As seen in Fig. 1A, the ratio of IFN-γ- to IL-4-secreting cells was approximately 10 and was even higher after a second vaginal challenge. Likewise, in mice that were first given live MoPn via the intranasal or oral route and then challenged with MoPn vaginally, the ratio of IFN-γ- to IL-4-secreting cells was greater than 10. In contrast, mice given live MoPn subcutaneously produced a ratio of

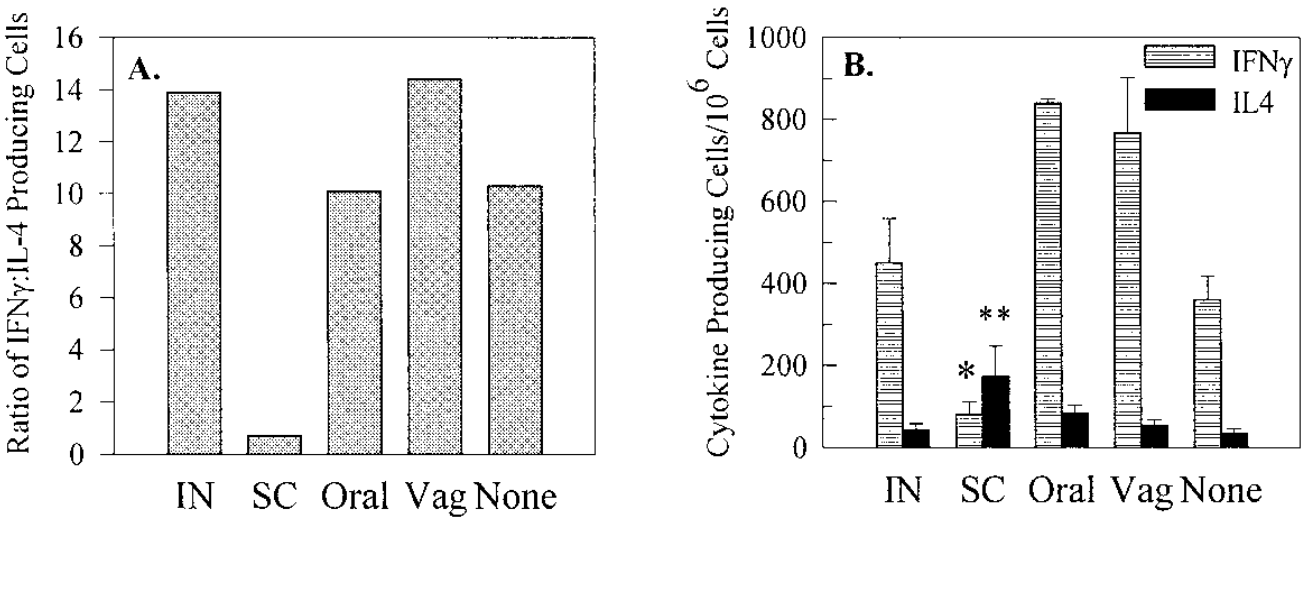

FIG. 1. Comparison of the cytokine profiles in the ILN following different routes of live-MoPn administration. Mice were given live MoPn by various routes and then vaginally challenged with live MoPn. The ILN from groups of 10 mice were harvested and pooled 7 days after challenge. (A) The ratio of IFN-γ-producing cells to IL-4-producing cells was determined from the frequency of cytokine-producing cells obtained with the ELISPOT assay. Data are expressed as the mean ratio from multiple experiments (numbers of experiments are given): intranasal (IN), two; subcutaneous (SC), two; vaginal (VAG), five; and none, two. Oral delivery of MoPn was performed only once, and data are expressed as a mean of that experiment. (B) The frequencies of IFN-γ-producing cells and IL-4-producing cells were determined from the means of triplicate wells with the ELISPOT assay. Data are expressed as the mean frequency ± SEM from multiple experiments. *, significantly lower frequency compared with the vaginal group ($P < 0.05$; one-way ANOVA and t test); **, significantly greater frequency compared with the vaginal group ($P < 0.05$; one-way ANOVA and t test).

IFN-γ- to IL-4-secreting cells that was less than 1.0. Thus, the route of MoPn administration appears to influence the local T-cell cytokine pattern after vaginal challenge, with mice given live MoPn vaginally, orally, and intranasally developing a Th1-type response, while those given live MoPn subcutaneously develop a Th2-dominant response.

When the frequency of IFN-γ-secreting cells was compared between groups given live MoPn by different routes, mice given live MoPn via the subcutaneous route had detectable but significantly smaller numbers (Fig. 1B, $P < 0.05$) of IFN-γ-secreting cells than that observed in mice first given live MoPn vaginally. Surprisingly, mice receiving only a single MoPn vaginal inoculation had greater levels of IFN-γ compared with those for mice receiving live MoPn subcutaneously followed by a vaginal challenge. However, the frequency of IL-4-producing cells was significantly increased in the subcutaneous group (Fig. 1B, $P < 0.05$). Thus, subcutaneous administration appeared to shift the nature of the immune response to MoPn following a vaginal challenge to a Th2-type cytokine pattern, with a significantly lower frequency of IFN-γ-secreting cells and increased numbers of IL-4-secreting cells.

In a natural infection, chlamydiae preferentially target mucosal epithelial cells, and it was not clear whether subcutaneous injection of live MoPn produced an infection at that site. Since this could influence the difference in cytokine profiles between mucosal routes of MoPn administration and the subcutaneous route, we cultured swabs and cell scrapings from the site of subcutaneous injection. By injecting 1×10^8 IFU of live MoPn, we were able to recover MoPn from both swabs and skin scrapings of three mice 6 days after injection (mean ± standard error of the mean [SEM]: 3,245 ± 2,696 and 4,733 ± 4,011 IFU/ml, respectively). All mice examined were culture positive, but the number of organisms recovered was smaller than that recovered from cervico-vaginal swabs of vaginally inoculated mice (see Fig. 4). The infection was sustained for at least 13 days after injection with 92 ± 50 IFU/ml (mean ± SEM) recovered from swabs and 611 ± 154 IFU/ml recovered from skin scrapings of three mice. Interestingly, no chlamydiae were recovered from scrapings of muscle cells underlying the injection site. These data support the contention that the route of initial MoPn delivery and not active infection determines the type of Th cell cytokine profile.

Administration of UV-inactivated MoPn. Sufficient levels of IFN-γ appear to be a requisite for resolution of an MoPn vaginal challenge, since in mice, a Th1-type response is protective and *C. trachomatis* is susceptible to killing by IFN-γ. Furthermore, the production of a protective Th1 immune response to *C. trachomatis* may depend on an initial infection with viable organisms. To address this issue, we examined the cytokine profile following administration of UV-inactivated MoPn instead of live MoPn by intranasal, oral, and subcutaneous routes. Figure 2A shows that the ratio of IFN-γ to IL-4 in the ILN was 12.7 for mice first given UV-inactivated MoPn via the intranasal route and then challenged with live MoPn vaginally. This ratio was similar to that found with mice given live MoPn by the same route (Fig. 1A). Likewise, orally administered UV-inactivated MoPn (50 μg) also produced a predominant Th1-type response (Fig. 2A). In contrast, the same dose of UV-inactivated MoPn (50 μg) delivered subcutaneously produced a predominant Th2 cytokine profile with a higher frequency of IL-4- than IFN-γ-producing cells (Fig. 2). Thus, initial administration of UV-inactivated MoPn produced the same predominant cytokine pattern as that seen when live MoPn was given by that particular route.

Although the ratios of IFN-γ to IL-4 for each route of administration were similar for live and UV-inactivated MoPn,

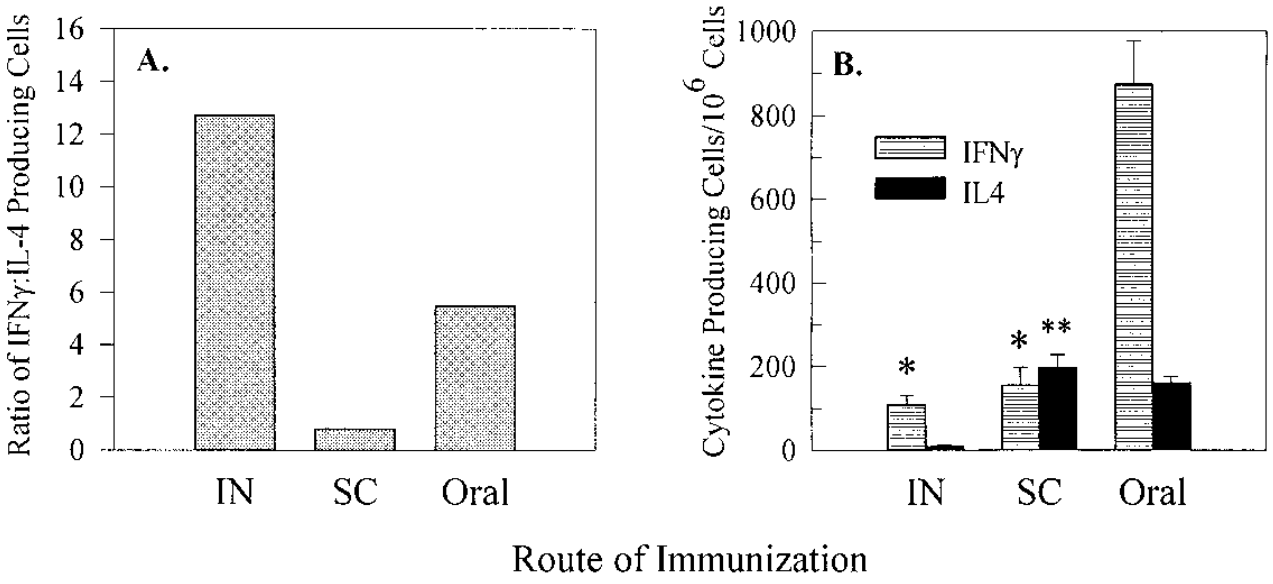

FIG. 2. Comparison of the cytokine profiles in the ILN following different routes of UV-inactivated MoPn administration. Mice were given UV-inactivated MoPn by various routes and then vaginally challenged with live MoPn. The ILN from groups of 10 mice were harvested and pooled 7 days after challenge. (A) The ratio of IFN-γ-producing cells to IL-4-producing cells was determined as described in the legend to Fig. 1. (B) The frequencies of IFN-γ-producing cells and IL-4-producing cells were determined as described in the legend to Fig. 1. *, significantly lower frequency compared with the vaginal group (Fig. 1) ($P < 0.05$; one-way ANOVA and *t* test); **, significantly greater frequency compared with the vaginal group ($P < 0.05$; one-way ANOVA and *t* test). IN, intranasal; SC, subcutaneous.

the frequencies of both IFN-γ and IL-4 were decreased in mice given UV-inactivated MoPn by the intranasal route (Fig. 2B). Furthermore, the frequency of IFN-γ-producing cells in these mice was significantly diminished ($P < 0.05$). However, the frequencies of IFN-γ- and IL-4-producing cells seen with mice given UV-inactivated MoPn by the subcutaneous and oral routes were similar to those observed following administration of live MoPn (Fig. 1B and 2B). Taken together, these data confirm that the route of initial MoPn delivery and not infection determines the type of Th cell cytokine profile.

Comparison of the anti-MoPn serum IgG isotype levels for different routes of administration. Another measure of the nature of the immune response is the pattern of IgG isotypes expressed in serum after antigen exposure. Serum IgG isotype expression in response to antigenic stimulation is influenced by the types of cytokines produced by T cells. For instance, IL-4 enhances IgG1 isotype switching, while IFN-γ suppresses IgG1 and enhances IgG2a levels in mice (8). We compared the MoPn-specific, serum IgG1 and IgG2a levels in mice following the administration of live and UV-inactivated MoPn by different routes. As seen in Fig. 3, mice given live MoPn subcutaneously had significantly higher titers of anti-MoPn IgG1 compared with the titers for those groups given live MoPn via vaginal and intranasal routes. The delivery of live MoPn orally produced a mean anti-MoPn IgG1 titer that was higher than those for the intranasal and vaginal groups but lower than that for the subcutaneous group and that did not differ statistically for any group. Thus, increases in anti-MoPn IgG1 titers in serum were associated with increases in the frequency of IL-4-secreting cells (Fig. 1B). No differences in anti-MoPn IgG2a titers were seen between groups, although the subcutaneous group had a significantly decreased frequency of IFN-γ-secreting cells (Fig. 1B). This finding is not unpredicted, since low doses of IFN-γ can enhance IgG2a production and anti-IFN-γ treatment can only partially reduce IgG2a levels (8). Serum anti-MoPn IgG2a but not IgG1 antibody levels were found to be significantly decreased in mice given UV-inactivated MoPn by the intranasal and oral routes compared with those in mice given live MoPn. Subcutaneous administration of UV-inactivated MoPn reduced the level of IgG1 but did not significantly change the titer of IgG2a. Therefore, anti-MoPn isotype titers in serum provide additional supportive evidence that prior subcutaneous administration shifts the cytokine pattern following MoPn vaginal infection from a characteristic Th1-like response to a Th2-like response.

The influence of the route of MoPn administration on protection from subsequent MoPn vaginal infection. Since the route of MoPn administration influenced the cytokine profile to MoPn following a challenge infection and resolution of an infection appears to correlate with a Th1 response (12), we examined whether different routes also influenced the resolution of a challenge infection. As seen in Fig. 4A, the majority of mice given live MoPn via intranasal, oral, and vaginal routes had resolved infection and were culture negative 15 days postchallenge. By comparison, the majority of mice in the subcutaneous group were not culture negative until day 18 postchallenge. Although the resolution of infection was significantly delayed, prior subcutaneous administration did provide protection compared with that for mice receiving only a single vaginal challenge. Subcutaneously injected mice resolved the challenge infection earlier than mice following a primary vaginal challenge, with 89% of this group becoming isolation negative on day 18 compared with only 23% of the group receiving only a single vaginal challenge (Fig. 4A).

As seen in Fig. 4B, mice which were given a second genital infection with MoPn also shed less chlamydiae than mice following a single infection. Likewise, mice given live MoPn via the intranasal and oral routes also had significantly lower levels of MoPn shedding as early as 3 days after challenge infection. While mice given live MoPn subcutaneously had threefold-smaller numbers of chlamydiae recovered from their swabs compared with that for controls early after infection, the levels of shedding were not significantly lower ($P < 0.05$) until day 9. Thus, the route of administration did appear to influence the

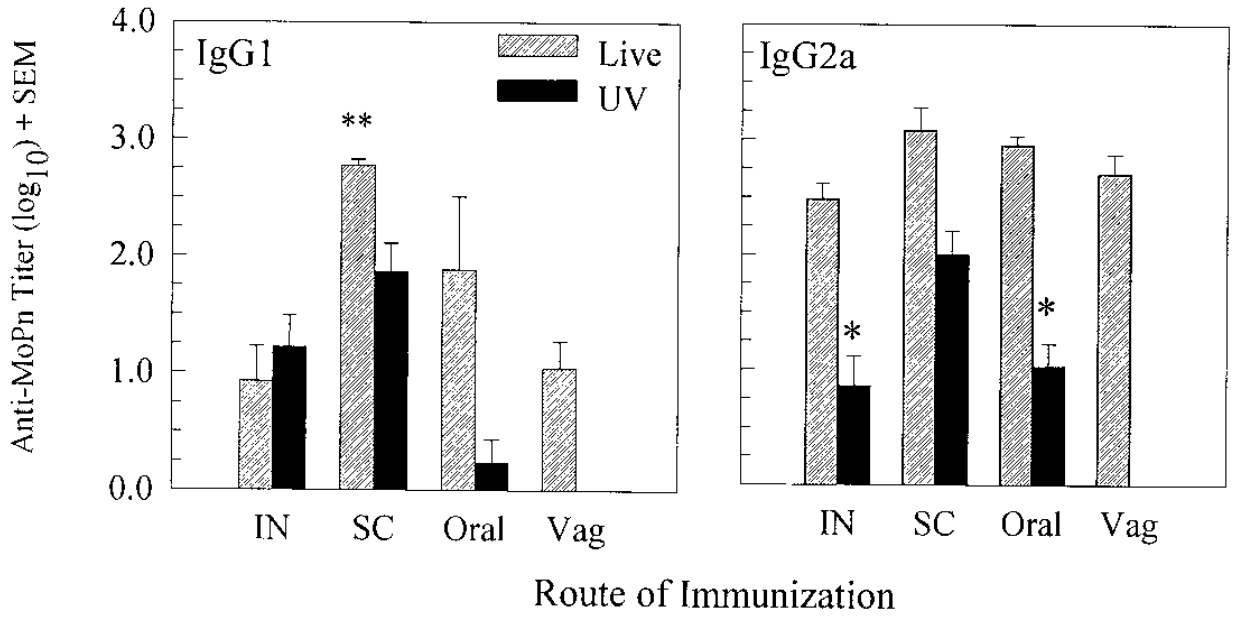

FIG. 3. Comparison of the serum anti-MoPn IgG1 and IgG2a titers induced by live and UV-inactivated MoPn by various routes. Sera were collected from mice 2 to 3 weeks after the second exposure to live or UV-inactivated MoPn. The anti-MoPn IgG1 (left panel) and IgG2a (right panel) titers were determined by ELISA. Data are expressed as the mean titer (log_{10}) ± SEM for each group from multiple experiments (numbers of experiments are given): intranasal (IN), two; subcutaneous (SC), two; oral, one; vaginal (VAG), five. The total number of mice for each group is as follows: intranasal, 12; subcutaneous, 10; oral, 4; and vaginal, 24. **, significantly higher titers compared with the oral and vaginal groups ($P < 0.05$; one-way ANOVA and *t* test); *, significantly lower titers compared with the vaginal group ($P < 0.05$; one-way ANOVA and *t* test).

degree of protection, with the subcutaneous route conferring less protection.

We also examined the ability of UV-inactivated MoPn delivered intranasally, orally, or subcutaneously to protect mice from a subsequent vaginal challenge with live MoPn. We found that neither the rate of recovery from MoPn infection nor the amount of chlamydiae shed was diminished by any route (data not shown). This finding was surprising, since mice given UV-inactivated MoPn, both intranasally and orally, produced a predominant Th1-type response (Fig. 2). Apparently, exposure

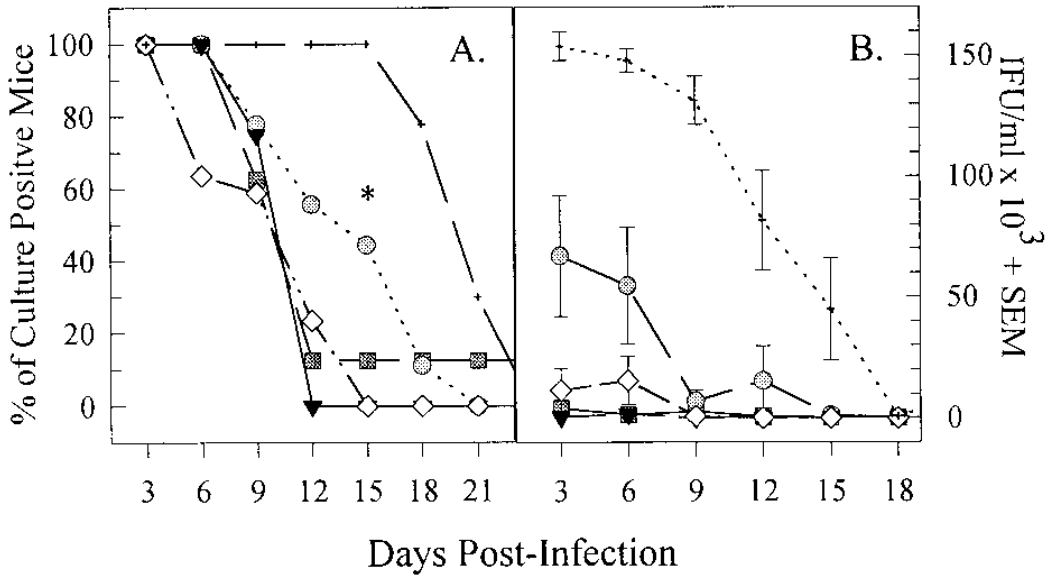

FIG. 4. Infectivity rate and magnitude of MoPn shedding after vaginal inoculation following administration of live MoPn by various routes. Mice were given live MoPn by various routes and then vaginally challenged with live MoPn. Beginning the third day after MoPn challenge, mice were monitored for MoPn shedding in the genital tract with cervico-vaginal swabs collected every third day. Data are expressed as the percentages of mice that were culture positive (A) and levels of MoPn shedding (B) for each group. Data are pooled from multiple experiments (numbers of experiments are given): intranasal, two; subcutaneous, two; oral, one; vaginal, five; none, two. The total number of mice for each group is as follows: intranasal, five to eight; subcutaneous, nine; oral, four; vaginal, 18 to 23; none, 5 to 10. *, a significantly higher percentage of mice were culture positive 15 days after infection compared with the vaginal control group ($P < 0.01$; Fisher's exact test). ▨, intranasal; ◍, subcutaneous; ▼, oral; ◇, vaginal; +, none.

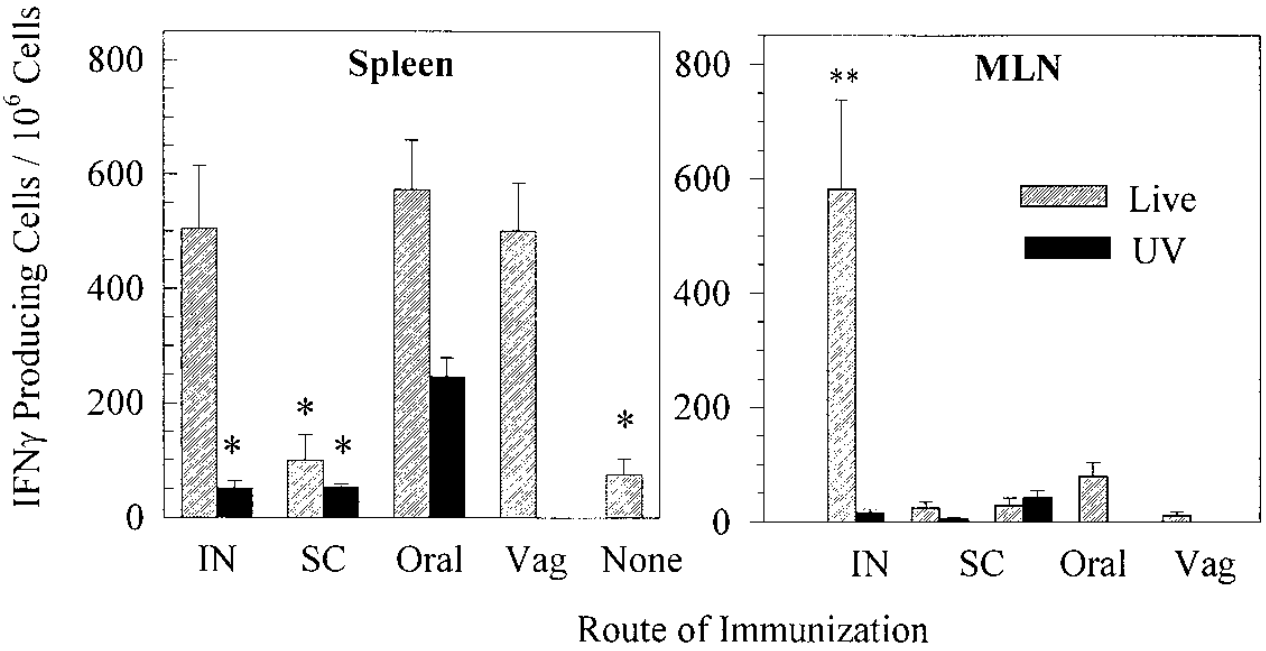

FIG. 5. Comparison of the frequencies of IFN-γ-secreting cells in the spleen and MLN for different routes of administration with live and UV-inactivated MoPn. Mice were given live or UV-inactivated MoPn by various routes and then vaginally challenged with live MoPn. The spleens and MLN from groups of 10 mice were harvested and pooled 7 days after challenge as described in the legend to Fig. 1. *, significantly lower frequencies compared with the vaginal group ($P < 0.05$; one-way ANOVA and *t* test); **, significantly higher frequency compared with the other groups ($P < 0.05$; one-way ANOVA and *t* test). IN, intranasal; SC, subcutaneous; VAG, vaginal.

to live MoPn appears to be essential for developing a protective immune response to the vaginal dose of MoPn used in these experiments for challenge infection and suggests that the development of a protective immune response also depends upon additional factors that are produced or associated with viable MoPn and not solely on IFN-γ production.

Distribution of the immune response to MoPn in various lymphoid tissues. The route of administration may influence the distribution of MoPn-specific lymphocytes throughout secondary lymphoid tissues which, in turn, may contribute to protection. To examine whether administration of MoPn at a local site can produce MoPn-specific lymphocytes which are found in systemic circulation, we analyzed the MoPn responses in the spleen. As can be seen in Fig. 5, anti-MoPn IFN-γ-producing cells were found in the spleen in all groups at similar to slightly lower frequencies compared with those found in the ILN. Furthermore, mice first given live MoPn vaginally, orally, or intranasally and then challenged vaginally produced a greater frequency of IFN-γ- to IL-4-secreting cells, ranging from 4 to 13 (data not shown). Mice given live MoPn subcutaneously had lower frequencies of IFN-γ-producing cells in the spleen (Fig. 5) and greater frequencies of IL-4-secreting cells compared with IFN-γ, with a IFN-γ-to-IL-4 ratio of less than 1 (data not shown). These data indicate that the cytokine profile produced by live MoPn exposure in a certain anatomical site remains stable even after MoPn-specific T cells have migrated to other systemic lymphoid tissues.

Also shown in Fig. 5 is the frequency of IFN-γ-secreting cells appearing in the spleen in response to live MoPn challenge following exposure to UV-inactivated MoPn. The frequency of IFN-γ-secreting cells in the spleen was significantly reduced in mice given UV-inactivated MoPn intranasally. While the frequency of IFN-γ-secreting cells was diminished in mice given UV-inactivated MoPn orally, the levels were not significantly different from that found with the vaginal control group. As predicted, the IFN-γ levels of mice given UV-inactivated MoPn subcutaneously were low and similar to the levels found in mice given live MoPn subcutaneously. No significant alterations in the frequency of IL-4-secreting cells were seen for any group (data not shown). The changes in IFN-γ production seen within the spleen when UV-inactivated MoPn was used were consistent with the changes observed in the ILN.

The genital tract is considered part of the mucosal immune system (18). Therefore, we also examined dissemination of the MoPn response to secondary lymphoid tissues associated with the mucosal immune system, such as the MLN. As reported previously, IFN-γ-secreting cells can be detected in the MLN following a second MoPn inoculation (4). However, we observed a strikingly higher frequency of IFN-γ-secreting cells in the ILN following intranasal administration with live MoPn. Also, the frequency of IFN-γ-secreting cells found in the MLN was significantly reduced after intranasal administration of UV-inactivated MoPn. IL-4-secreting cells were also detected in the MLN; however, the frequencies were very low (<20 per 10^6 cells; data not shown) for all groups. Again, a predominant Th1-type response was seen in the MLN from mice given live or UV-inactivated MoPn intranasally, orally, or vaginally, with ratios of IFN-γ to IL-4 of greater than 15 (data not shown). Increased proliferative responses to MoPn were observed in the MLN following intranasal and oral administration of live MoPn compared with those observed for mice given MoPn vaginally (data not shown). Taken together, these data show that the cytokine pattern produced after MoPn administration at a particular anatomical site remains consistent even after the cells migrate to other secondary lymphoid tissues. Furthermore, routes which provide protection from an MoPn vaginal challenge, intranasal and oral, are associated with the production of IFN-γ-secreting cells throughout the systemic and local arms of the immune system.

DISCUSSION

In this study, we have demonstrated that the local tissue microenvironment or draining lymph node where an immune response is initiated influences the type of T-cell response, Th1 or Th2, that develops at a distant local site. Moreover, we have linked the development of a local protective Th1-type response to the route of initial MoPn administration. First, we have shown that introduction of live MoPn via vaginal, oral, and intranasal routes produces a protective, Th1-dominated, immune response upon vaginal challenge. Second, the local immune response to an MoPn vaginal challenge can be shifted from the characteristic Th1-type response to a Th2-type response by first exposing mice to live MoPn by subcutaneous injection. These mice were found to have a reduced frequency of IFN-γ-secreting cells, an increased frequency of IL-4-secreting cells, and increased levels of anti-MoPn IgG1. The inability of a subcutaneous injection to produce a Th1 response does not appear to be due to an inability of that site to support MoPn infection. In addition, we have shown that a shift in the immune response to a dominant Th2-type response results in a diminished capability for resolving a vaginal challenge infection. Thus, the route of initial administration alone can have a significant effect on protection from a subsequent local MoPn vaginal infection.

Although the route used for antigen delivery or site of infection (22) can sway a developing immune response toward a Th1 or Th2 type of response, the mechanisms controlling this are not well understood. Recent evidence has suggested that the type of antigen presenting cell (APC) present at the site of injection can influence T-cell cytokine production (7, 9). On the other hand, others have demonstrated that the transfer of different types of APC pulsed with antigen produces the same type of response (delayed-type hypersensitivity or antibody) when injected at the same site (20). This suggests that environmental factors present within lymphoid tissues at the time T cells are activated, and not the type of APC, are important in directing cytokine production. These studies differed somewhat from our findings, since the authors showed that subcutaneous injection of antigen-pulsed macrophages and Langerhans cells produced a Th1-type response (delayed-type hypersensitivity), while we found a Th2-dominated response after mice were injected with live MoPn subcutaneously. However, we used an intact organism and not soluble antigen, which may elicit other factors in the local microenvironment that could direct T-cell cytokine production.

The local tissue environment can determine the type of immune cells which have access to that site through the selective expression of vascular addressin molecules on high endothelial venules (10). Exclusive expression of these molecules is responsible for the differential lymphocyte migration seen between mucosal and peripheral lymphoid tissues (25). These molecules as well as VCAM-1, a vascular addressin which supports lymphocyte trafficking to sites of inflammation, can also be induced on nonlymphoid endothelial cells by inflammatory cytokines (10, 35). The specific ligands for these vascular addressins are selectively induced on lymphocytes, depending on the site of T-cell activation (17). Following a primary MoPn vaginal infection, T-cell proliferative responses against MoPn were found in the ILN and MLN but not in the cervical lymph nodes (4). Thus, T cells activated in lymph nodes draining the site of subcutaneous injection (cervical lymph nodes) may not be able to enter the vaginal mucosa in numbers large enough to effectively prevent infection. Alternatively, Th2-type T cells may not have the ability to access certain tissues as readily as Th1-type T cells (30).

The interaction of viable chlamydiae with the local environment appears to be necessary for forming a protective immune response, since mice given UV-inactivated MoPn were unable to reduce the course of infection or the amount of MoPn shed. IFN-γ has been associated with a protective immune response to MoPn; however, the frequency of IFN-γ-producing cells was significantly diminished in mice only following intranasal but not oral administration of UV-inactivated MoPn. Thus, factors other than a polarized Th1 or Th2 cytokine profile may be involved in producing a protective immune response to MoPn. Possibly the differentiation of live MoPn elementary bodies to the reticular body stage or enzymatic modification by host cells (11) may reveal immunodominant antigens not normally accessible to APC for processing and presentation. Also, surface antigens present on live MoPn may alter the costimulatory molecules present on APC which influence their ability to activate T cells (13). Alternatively, injection of live MoPn, which can replicate, may produce more antigen available to the immune system than injection of UV-inactivated MoPn. While the dose of antigen has been shown to alter the cytokine profile (7), in our system this does not seem likely, since the same dose of UV-inactivated MoPn delivered orally and subcutaneously produced different cytokine profiles.

The implications for development of a vaccine are obvious, and successful strategies have been demonstrated with some animal models. In the guinea pig:guinea pig inclusion conjunctivitis (GPIC) model where both antibody and cell-mediated immunity are critical for resolution of and resistance to genital infection, subcutaneous delivery of GPIC has been shown to elicit a protective response, while repeated vaginal infections caused enhanced pathology (29). In this report, prior subcutaneous injection did not provide the degree of protection conferred by other routes. However, the mice did show a reduced course of infection and shedding following vaginal challenge compared with that seen with mice following a primary vaginal challenge. It has also been demonstrated in the murine and primate systems that the immune response can elicit a pathologic response to repeated infection. While it may be improbable to develop a vaccine that would completely protect from infection, it may be possible to limit the infection to the lower genital tract, preventing upper tract sequelae. In support of this, we have recently demonstrated with the guinea pig:GPIC model that it is possible to reduce upper tract pathology by prior subcutaneous immunization with UV-inactivated GPIC (unpublished data). Thus, an immunization protocol that elicits a less-polarized Th1 response but still produces a shortened infection, such as the subcutaneous route, may prove to be the most optimal for vaccination.

In this report, we focused on the initial route of MoPn exposure and its ability to influence the nature of the immune response and protective immunity. However, the use of adjuvants can also alter immune responses to the same dose of antigen at the same anatomical site (3). For instance, the immune response following mucosal immunization with a vector expressing tetanus toxoid can shift the preferential expression of a Th2 response to a Th1 response (40). In order to develop efficacious vaccines, a better understanding of the factors that shape natural immunity to individual organisms is needed, as well as methods to exploit beneficial immunity while reducing pathologic responses.

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