Susceptibility to Reinfeciton after a Primary Chlamydial Genital Infection Is Associated with a Decrease of Antigen-Specific T Cells in the Genital Tract

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Received 24 September 1990/Accepted 9 January 1991

We tested the hypothesis that the intensity of specific antichlamydial T cell-mediated immunity in the genital tract of female guinea pigs infected intravaginally with the chlamydial agent of guinea pig inclusion conjunctivitis would determine the resistance or susceptibility to reinfection after a primary chlamydial infection. T cell-enriched lymphocytes were isolated by collagenase treatment of genital tract tissues from either infected or control uninfected female guinea pigs at various times after infection. The nylon wool-enriched T lymphocytes were evaluated for expression of antigen-specific T cell-mediated immunity in vitro by using a blast transformation assay. Both uninfected and infected genital tracts contained T cells, as evidenced by reactivity to concanavalin A, although a greater number of T lymphocytes was detected in the genital tracts of infected animals compared with that in controls. Significant antigen-specific T-cell activity could be detected in the genital tract tissue by 7 days after a primary genital tract infection with the chlamydial agent of guinea pig inclusion conjunctivitis. When antigen-specific activity was assessed at different times after infection, the intensity of the response of genital tract-associated T lymphocytes was directly proportional to the degree of resistance of the animals to genital challenge. Thus, susceptibility of animals to reinfection by chlamydiae appears to be associated with the intensity of the local T cell-mediated immune responses in the genital tract of infected animals.

Chlamydial infection of the genital tract is associated with disease in women that can progress to severe complications, including chronic inflammatory disease, fallopian tube damage, infertility, and potentially ectopic pregnancy (5). After a primary chlamydial genital infection and its resolution, some evidence suggests that there is a transient period of acquired immunity when the individual is apparently resistant to a challenge infection. However, the individual shortly becomes susceptible to reinfection (7).

This short duration of immunity has also been documented in the guinea pig (21) and marmoset (6) models of genital infection. It has not been possible to identify any one factor that could be identified with susceptibility to reinfection, since both serum and local antibody responses and systemic levels of antigen-specific T cells all decrease at the time that animals can be reinfected (21). The reason for this lack of long-term protective immunity is unknown. Previous reports in the guinea pig system have indicated that both humoral and T cell-mediated immunity are required for the resolution of a chlamydial genital tract infection and that both mechanisms are also necessary for resistance to reinfection (18, 19, 22, 23). Studies in the murine system of chlamydial genital infection with the agent of mouse pneumonitis have revealed that T cell-mediated immunity is obligatory for chlamydial control, since B cell-deficient mice are able to resolve the infection and are immune to challenge infection (17). Furthermore, nude mice, which exhibit persistent chlamydial infection after a primary genital infection, can be cured by the adoptive transfer of T lymphocyte-enriched spleen cells from chlamydia-immune syngeneic animals (17a).

In previous studies the functional state and the number of local genital tract-associated T cells as the determinants of chlamydial resistance were not considered. Although genital tract-associated T lymphocytes have been identified (3, 15), their functional capabilities remain undefined. Since cell-mediated immunity is essential for resistance to chlamydial reinfection but the peripheral blood T-cell response alone could not account for antichlamydial resistance, the present studies investigated the role of guinea pig genital tract-associated antigen-specific T lymphocytes in the resistance to chlamydial genital infection. The results suggest a crucial role for local genital tract-associated T lymphocytes in the resistance of guinea pigs to chlamydial genital infection, revealing that the genital tract contains immunocompetent T cells and that susceptibility to reinfection appears to be due to a time-dependent decrease of the intensity of antigen-specific T lymphocytes in the genital tract mucosa.

MATERIALS AND METHODS

Animals. Female guinea pigs of the Hartley strain and weighing 450 to 500 g were procured from Sasco Laboratory, Omaha, Nebr. All animals were housed individually and maintained with food and water ad libitum. The room was maintained on a cycle of 12 h of light and 12 h of darkness.

Chlamydial strain and infection protocols. The chlamydial agent of guinea pig inclusion conjunctivitis (GPIC) employed for infection was grown in McCoy cells and prepared as previously described (21). Animals were infected by intravaginal inoculation of 0.05 ml of GPIC suspension in sucrose-phosphate buffer (pH 7.2) containing 10^7 inclusion-forming units per animal. The course of the infection was assessed as was previously described (21) by determining the percentage of cells bearing chlamydial inclusions on Giemsa-
of chlamydiae from cervical swabs.

Preparation of genital tract-associated T lymphocytes. Animals were anesthetized, and the genital tract between the vagina and ovaries (i.e., the cervix, uterus, and fallopian tubes) was excised, weighed, and placed in sterile HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Gibco Laboratories, Grand Island, N.Y.)-buffered RPMI 1640. Each explant was washed further in buffered RPMI 1640 and placed in 7 ml of 0.6-μg/ml filter-sterilized type I collagenase (Sigma Chemical Co., St. Louis, Mo.). The tissue was minced thoroughly in this solution with surgical scalpels and incubated at 37°C for 45 to 60 min. After incubation, the tissue was further minced and teased with forceps. The teased material was filtered over a sterilized nylon screen gauze with an additional 30 to 40 ml of buffered RPMI 1640. The cells were washed three times with the buffered medium.

Enrichment for nylon wool-nonadherent T cells was carried out by the method of Weinblatt and coworkers (27). Briefly, collagenase-isolated cells were washed three times and suspended in 2 ml of HEPES-buffered RPMI 1640 supplemented with 5% fetal calf serum. The suspension was then layered over a sterilized nylon wool column and washed into the column with an additional 2 ml of the carrier medium. The column was incubated at 37°C for 45 to 60 min, and nonadherent T cells were eluted with 40 ml of the supplemented medium. The cells were washed and suspended in complete RPMI 1640 medium, consisting of RPMI 1640 (Hazelton Research Products, Denver, Pa.) supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 1% glutamine, 1% HEPES, 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (all from Gibco) and 2 × 10⁻⁵ M 2-mercaptoethanol (Sigma), counted, and adjusted to the required concentration for cultures. The absolute yield (i.e., the total number of cells per animal) and specific yield (i.e., the number of cells expressed per gram of genital tissue) of T lymphocyte-enriched cells from the genital tract by the foregoing procedure vary with the immune status of the animals (immune versus normal), time after genital infection, and number of immunizations (primary versus secondary infection). Typically, the genital tract of a normal guinea pig yielded between 0.4 × 10⁶ and 1.0 × 10⁷ T lymphocyte-enriched cells, whereas an immune animal produced a range of 1.0 × 10⁷ to 5.0 × 10⁷ T lymphocyte-enriched cells. Trypan blue exclusion revealed that the cells prepared by this method were >95% viable.

To normalize the cell count data for all groups of animals (normal, immune, resistant, and susceptible groups) employed in these studies and for the differences in the weights of the genital tracts obtained from the different animals, the genital tract T-cell count was expressed as the number of T lymphocyte-enriched cells obtained per gram of genital tract tissue. The T-cell number per gram of tissue was calculated by dividing the total nylon wool-purified cells from a genital tract explant by the weight of the tissue.

Preparation of antigen-presenting cells. Since we employed noninbred strains of guinea pigs in these studies and because evidence from our laboratory showed allogeneic responses among these guinea pigs in mixed leukocyte cultures (unpublished data), we prepared both the responding genital T cells and the antigen-presenting cells from the same animal. Spleens of individual guinea pigs were separately teased with forceps in Hanks medium. The single-cell suspension was treated with Tris-buffered ammonium chloride to lyse the erythrocytes, washed three times, and irradiated with 2,000 rads of X irradiation from a cesium source. The irradiated cells were washed again, counted, and suspended in complete RPMI 1640 medium.

T-cell proliferation assay. Responding T lymphocyte-enriched cells (10⁴ to 10⁵ cells per well) prepared from the genital tracts of female guinea pigs were seeded into triplicate cultures of 96-well tissue culture plates with 2 × 10⁵ autologous antigen-presenting cells. Experimental cultures also had 5 μg of HeLa-grown, UV-inactivated GPC per ml and all wells were adjusted to 0.20 ml (final volume). Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C for 5 days. T-cell activation was assessed by a tritiated thymidine incorporation assay (1). Briefly, on day 4 of incubation, cultured cells were pulsed with 1 μCi of [³H]thymidine for 16 to 24 h before the end of the incubation period. At the end of incubation all cultures were harvested on filters, and the radioactivity associated with each culture was measured with a scintillation counter.

Data are expressed either as counts per minute, which measures the absolute response of the cells in cultures, or as the response per gram of tissue, which measures the intensity of antigen-specific T cells in the genital tract tissue.

Since different amounts of cells were obtained from individual genital tracts and different numbers of cells were used in cultures, depending on the cell yield, we found that expressing the activation measurement data as the intensity of antigen-specific T-cell responses for each genital tract normalized the conditions of the studies, produced a proper picture of the frequency and intensity of antigen-responsive T cells from each genital tract and enabled us to compare the responses from different animals.

The response per gram of tissue is given by the following: (experimental cpm − control cpm)/(cells per culture) × cells per gram of tissue.

RESULTS

Presence of antigen-specific T lymphocytes in the genital tract mucosa of genitally infected animals. The role of local genital mucosal T cells in immune responses elicited by genital infection has not been defined. To determine whether antigen-specific T cells could be isolated from the genital tracts of genitally infected guinea pigs, we prepared T lymphocyte-enriched cells from genital tract tissue by collagenase digestion. The isolated T lymphocyte-enriched cells were tested for T-cell function in mitogenic and antigen-specific T-cell-mediated immune responses by in vitro restimulation in a blast transformation assay. At 2 weeks after genital infection with GPC, the T-cell-enriched population prepared from the genital tract expressed an antigen-specific secondary immune response in vitro (Fig. 1A). Thus, whereas the normal (uninfected) genital tract T cells produced a mean response of 3,021 cpm, T cells from genitally immunized animals had a response of 20,293 cpm. The difference in response of primed and unprimed genital tract T cells to GPC in culture was statistically significant as determined by the one-tailed t test (P < 0.0019). The response of both cell populations to concanavalin A, a T-cell mitogen, indicates that both the infected and uninfected genital tracts contain T cells (Fig. 1B), although the response in infected animals was significantly greater (P < 0.0003). The data indicate that there is a resident population of immunocompetent T cells in the genital tract of female guinea pigs.
Relationship between local antigen-specific T cell-mediated response and susceptibility to chlamydial reinfection. The next question concerned the role of such a local genital tract T-cell response in protective immunity and/or in the short-term immunity observed in chlamydial genital infections. Based on the observation that the genital tracts of infected guinea pigs contain a population of antigen-specific T cells, we attempted to determine whether the level of local T cell-mediated immunity might be related to the resistance of the animals to secondary chlamydial infection. Our approach to investigating this was to determine the intensity of local T cell-mediated immunity of genital tract explants from three groups of female guinea pigs: (i) the first group consisted of normal (uninfected) animals, (ii) animals in the second group were infected 30 days previously and had resolved the infection, and (iii) members of the third group were infected 75 days previously and had also resolved the infection. It has been previously demonstrated that animals are resistant to reinfection at 30 days but become susceptible by day 75 (21). Figure 2 shows the responses per gram of tissue of normal (uninfected) guinea pigs and of guinea pigs infected 30 and 75 days previously. The data indicate that there is a high intensity of antigen-specific T cell-mediated immunity in the genital tract of animals that are resistant to reinfection (30 days) but that the immunity is significantly reduced for normal or previously infected but susceptible animals (day 75). The data were significant according to a one-way analysis of variance ($P < 0.0014$). When a Scheffé test for groups with significant differences was performed, the immunity of animals in the day 30 group was significantly greater than that of either the control group ($P < 0.0037$) or the day 75 group ($P < 0.0057$). However, there was no significant difference in immunity between the control and day 75 groups.

Kinetics of antigen-specific T-cell response in the genital tract after genital infection with GPIC. We next wanted to investigate the kinetics of antigen-specific T-cell proliferative response in the genital tract after a primary GPIC genital tract infection. The aim was to determine a possible relationship between the intensity of local antigen-specific T cell-mediated immunity and the course of chlamydial disease in female guinea pigs.

Groups of female guinea pigs were genitally infected with GPIC. On different days postinfection, vaginal scrapings were obtained, and the course of the infection was determined by inclusion scores. At the same time, T cells were prepared from the genital tract tissue of the animals, and the intensity of antigen-specific T-cell response was assessed. The intensity of antigen-specific T-cell response in the genital tract is intimately associated with the course of GPIC genital infection in the guinea pig (Fig. 3). Thus, although the response became detectable by 1 to 2 weeks after genital infection, the resolution of the infection by the animals was coincident with the peak of high-intensity T cell-mediated immunity in the genital tract on day 21. Elevated T-cell response was still evident by day 30, although this was lower than the day 21 peak response. By 75 days postinfection, when animals are susceptible to reinfection, the intensity of antigen-specific T-cell response had essentially decreased to baseline levels. Thus, the pattern of expression of local genital T-cell response after genital infection with GPIC appears to be directly related to the ability of the animal to resolve the infection and maintain a temporary resistance to reinfection.

It was of interest to know whether there is also a relationship between resistance or susceptibility to reinfection and the number of T lymphocyte-enriched cells isolated from the genital tract after genital infection with Chlamydia. To determine this, we calculated the number of T lymphocytes
VOL. 59, T-cell response represents the separate five animals in expressing curves an T-cell number scores tion genital the number of T lymphocytes the isolated from the genital tract antigen-specific T after genital infection with GPIC. infected with GPIC 7, 14, 21, 30, and 75 days previously were enumerated, and the total counts were expressed as total T cells per gram of genital tract tissue, which measures the intensity of T cells in the genital tract. Each data point in the T-cell count curve represents the mean response plus the standard error of the mean for five independent experiments conducted on five animals in each group. Each data point measuring inclusion scores represents the mean plus the standard error of the mean for five separate animals in the group.

isolated from the genital tract from each group of animals in the above experiment. There is a close relationship between the number of T lymphocytes present in the genital tract after genital infection with GPIC and the ability of the animal to resist the infection (Fig. 4). The superimposition of the curves expressing the local kinetics of cells and the presence of an antigen-specific T-lymphocyte response (Fig. 3 and 4) after genital exposure to GPIC suggests that the increased T-cell number is related to the increased frequency of antigen-specific T cells in the genital tract.

Comparison of the intensities of genital T-cell responses of guinea pigs in primary and secondary genital infection with GPIC. A primary genital infection of guinea pigs takes approximately 3 weeks to resolve; when the animals become susceptible to reinfection by day 75, a secondary genital infection is resolved faster, with lower numbers of chlamydiae detected immediately after infection (21). On the basis of the foregoing studies, we hypothesized that a secondary infection might be associated with a more intense T-cell response in the genital tract. We therefore compared the extent of the genital T-cell response on different days after primary and secondary infection with GPIC. One group of guinea pigs was given a primary genital GPIC infection at the same time that a group infected 75 days previously was challenged. The intensity of T cell-mediated immunity in the genital tracts of these animals was determined on different days after the infection. Figure 5 demonstrates a trend, although not statistically supported (two-way analysis of variance) because of large variability between animals, that reinfection of guinea pigs is associated with a local genital tract T cell-mediated immune response that is more vigorous than the response to a primary infection. This phenomenon may play a role in the reduced infectivity and earlier resolution of a secondary infection.

**FIG. 3.** Kinetics of antigen-specific T-cell response in the genital tract after genital infection with GPIC. T lymphocyte-enriched cells from genital tracts of female guinea pigs that were vaginally infected with live GPIC 7, 14, 21, 30, and 75 days previously were stimulated in culture with UV-inactivated GPIC for 5 days, and T-cell activation was measured by [3H]thymidine uptake. The data are expressed as counts per minute per gram of tissue. Each data point measuring T-cell response represents the mean response plus the standard error of the mean for five independent experiments conducted on five animals in each group. Each data point measuring inclusion scores represents the mean plus the standard error of the mean for five separate animals in the group.

**FIG. 4.** Kinetics of T lymphocytes in the genital tract after genital infection with GPIC. T lymphocyte-enriched cells isolated from genital tract explants of female guinea pigs that were vaginally infected with GPIC 7, 14, 21, 30, and 75 days previously were enumerated, and the total counts were expressed as total T cells per gram of genital tract tissue, which measures the intensity of T cells in the genital tract. Each data point in the T-cell count curve represents the mean response plus the standard error of the mean for five independent experiments conducted on five animals in the group. Each data point measuring inclusion scores represents the mean plus the standard error of the mean for five separate animals in the group.

**DISCUSSION**

It has been previously demonstrated by clinical observations in humans and in the guinea pig-GPIC model of chlamydial genital infection that resistance to reinfection is relatively short lived (7, 21). The exact mechanism for this phenomenon is not clear, but studies from this laboratory have indicated that both antibody-mediated immunity and cell-mediated immunity are required for resistance to reinfection (18, 22). The data suggest that local immunoglobulin G in the genital tract may be responsible for reducing the level of a challenge infection (20), whereas cell-mediated immunity may be necessary to prevent reinfection or resolve a second infection (22). Preliminary data also showed that, in general, a lower number of local cervical mononuclear cells were present in guinea pigs with depressed cell-mediated immunity compared with that in immunologically intact animals (22). Animals with depressed cell-mediated immunity were less resistant to reinfection and were unable to resolve the challenge infection as readily. The role of the local T-cell response has been further pursued; in the current study, we demonstrate that functional T cells can be isolated from the guinea pig genital tract and that there is a strong association between the intensity of local genital T-cell response and the resistance to challenge infection.

The female genital tract is a component of the mucosal immune system (2, 12, 13, 15, 26) with immunocompetent cells found in the cervix and in the vagina (8, 11, 24, 25) and probably also in the uterus (8, 11, 24), and the presence of antigen or the estrous cycle can affect the preponderance of lymphocytes in these locations (10, 26, 30). Genital tract-associated T lymphocytes isolated from both normal and infected guinea pigs responded vigorously to the T-cell mitogen concanavalin A, although only cells from GPIC-primed animals responded to UV-inactivated GPIC in culture. The presence of functional T cells in both normal and infected genital tracts suggests that there are resident immu-
nocompetent T lymphocytes in the female genital tract mucosa which could play a significant role in antigen handling and immune reactivity in the local tissue and probably also in systemic immunity by their distribution to other lymphoid tissues of the body.

Studies of the intensity of local antigen-specific T-cell response in three groups of guinea pigs revealed that, although susceptible normal (uninfected) animals expressed little or no antigen-specific T-cell response, animals infected 30 days previously had an elevated local antigen-specific T-cell response. Interestingly, guinea pigs are markedly resistant to reinfection 30 days after infection but uniformly become reinfected when challenged 75 days after infection (21). These data indicate that, after exposure to chlamydiae, antigen-specific T cells appear in the genital tract of guinea pigs and that may play a significant role in the resolution of the infection and may also account for the temporary resistance to reinfection. However, after this transient resistance is a dampening of the local T-cell immunity that results in susceptibility to reinfection.

In kinetic studies, a close association was therefore established among three parameters evaluated: the intensity of local T-cell response, the course of chlamydial infection, and susceptibility to reinfection. The genital tract-associated antigen-specific T cells may be involved in the initial immune sensitization and may provide help for local antibody production and for the elicitation of T cell-mediated immune responses, all of which may combine to alter the infection course and establish an effective, although temporary, protective immunity. It was particularly interesting to find that a secondary and anamnestic T-cell response by genital tract T lymphocytes could explain the faster resolution of the infection after genital challenge of animals that were susceptible to reinfection 75 days after a primary infection.

Our findings indicate that to achieve a successful vaccination against chlamydial genital infection, an immunization regimen that takes into consideration the profile of local T-cell immune response will be required. Immunization with other agents via specific mucosal routes, including oral and intranasal, elicited only a transient cell-mediated immunity, prompting Ogra and coworkers (14) to suggest that specifically antigen-sensitized lymphocytes may migrate away from the site of primary immunization and dilute in the constant pool of circulatory lymphocytes. Certainly, in the case of chlamydial genital infection, as local antigen dissipates, the stimulus for maintaining antigen-specific T cells in the local area wanes, prompting the cells to leave the local tissue. On reinfection, a process for the generation and recruitment of the T cells from the peripheral blood or local lymph nodes to the genital tract is initiated. This takes several days, as evidenced by the kinetic studies, thus permitting an infection of 3 to 6 days, which is common for animals 75 days or more after infection. However, the intensity of the infection is always much lower than that of the primary infection, probably as a result of local immunoglobulin G antibody (20).

The exact mechanism by which local T cells effect resolution of the infection is not clear, although it has been demonstrated that gamma interferon has antichlamydial activity (4). Moreover, treatment of mice infected with MoPn in the respiratory tract with anti-gamma interferon has increased mortality, suggesting an in vivo role for gamma interferon (28). There is also evidence that other lymphokines, such as tumor necrosis factor alpha, released from macrophages stimulated by T cells, could be an effective host defense mechanism (29). The evidence for cytotoxic T-cell activity is minimal but cannot be completely ruled out (9, 16).

ACKNOWLEDGMENTS

We thank Anne T. Kidd and Beth Pack for their excellent technical assistance.

This study was supported by Public Health Service grant AI23044 from the National Institutes of Health.

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