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Role of Cell-Mediated Immunity in the Resolution of Secondary Chlamydial Genital Infection in Guinea Pigs Infected with the Agent of Guinea Pig Inclusion Conjunctivitis

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Guinea pigs which have recovered from a genital infection with the agent of guinea pig inclusion conjunctivitis demonstrate strong immunity to reinfection for a short period of time but then become susceptible to reinfection. The secondary infection is markedly shortened in duration and decreased in intensity. Previous studies have indicated an important role for humoral immunity in resistance to and in recovery from reinfection. However, the contribution of cell-mediated immunity to immunity toward or recovery from a secondary infection is not clear. Guinea pigs were infected in the genital tract with guinea pig inclusion conjunctivitis and were challenged at either 30 or 75 days after the primary infection. Prior to challenge, one group of animals was injected with rabbit anti-guinea pig thymocyte serum (ATS) while control groups received either normal rabbit serum or no treatment. Treatment was continued daily for the course of the experiment. On day 30, ATS-treated guinea pigs had a slightly higher rate of reinfection, and generally the infection persisted longer than in controls. On day 75, all animals became reinfected upon challenge, but control animals resolved their infections in 3 to 9 days. In contrast, most ATS-treated animals remained infected throughout the course of the experiment. Although the animals became reinfected, the levels of chlamydiae were much lower than those observed during the primary infection. ATS treatment abrogated T-cell responses, but serum and secretory antibody responses remained normal. Histopathological examination revealed some decrease in mononuclear infiltration of endocervical and uterine tissues in ATS-treated animals. These data indicate that previously infected guinea pigs require both cell-mediated immunity and humoral immunity for resolution of a challenge infection.

Studies with patients infected in the genital tract with *Chlamydia trachomatis* have indicated that both humoral and cell-mediated immune (CMI) responses are activated (3, 6, 8); however, the role of these responses in the resolution of the infection and in resistance to a second exposure have yet to be determined. Previous studies with guinea pigs infected in the genital tract with the chlamydial agent of guinea pig inclusion conjunctivitis (GPIC), a member of the species *Chlamydia psittaci*, have demonstrated that both antibody and CMI are required for resolution of a primary infection. When humoral immunity was abrogated by treating guinea pigs with cyclophosphamide, leaving CMI intact, animals were unable to resolve the infection and maintained an elevated level of infection for the duration of the experiment (16). When CMI was compromised prior to infection by treatment with anti-thymocyte serum (ATS), both serum and secretion antibodies developed and the infection accordingly decreased in intensity but did not resolve as long as ATS treatment was continued (13).

The role of antibody has also been examined in resistance to and recovery from a challenge infection (12). Guinea pigs deprived of humoral immunity by cyclophosphamide treatment were infected and then cured of the infection with tetracycline. Even though all of the animals had developed *Chlamydia*-specific CMI, they were still susceptible to reinfection when challenged by infection of the genital tract, indicating an important role for humoral immunity in resistance to reinfection. Moreover, the kinetics of the secondary infection in these immunologically compromised animals resembled that of a primary infection.

A recent study has indicated that complete resistance to reinfection in immunologically intact guinea pigs is relatively short-lived and that animals become reinfected when challenged as soon as 8 weeks after resolution of the primary infection (15). In this study, when the animals became reinfected, the course of the challenge infection was abbreviated and markedly lower in intensity, indicating that the animals did possess a significant degree of immunity. Thus, in the present study, we wanted to determine the extent, if any, to which this immunity was dependent upon CMI. To do this, female guinea pigs were infected in the genital tract with GPIC and were allowed to develop normal serum and secretion antibody responses as well as a normal CMI response. At either 30 or 75 days after resolution of the infection, animals were deprived of CMI by treatment with ATS, challenged with a fresh inoculum of GPIC, and then monitored for the course of the challenge infection.

MATERIALS AND METHODS

Experimental animals. Female Hartley strain guinea pigs, weighing 450 to 500 g, were obtained from Sasco Laboratories, Omaha, Nebr. All animals were housed individually in cages covered with fiber glass filters and were given food and water ad libitum. The room lighting was maintained on a 12 h light/12 h dark cycle.

Infection of guinea pigs with chlamydiae. GPIC for infection was grown in McCoy cell cultures (7). Portions of host cell-free chlamydiae were frozen in sucrose-phosphate

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buffer (2-SP) at −70°C until needed. Animals were infected by intravaginal inoculation with 0.05 ml of the GPIC suspension, which contained 1.3×10^7 (day 30) or 6.4×10^6 (day 75) inclusion-forming units (15). Animals were monitored for the course of the infection by assessing the number of chlamydial inclusions on a vaginal scraping stained with Giemsa and by the isolation of organisms in cell cultures from cervical swabs (15). The cultures were not quantitated but were merely scored as positive or negative for chlamydiae. The latter method has been found to be the more sensitive of the two assay systems (15). Whenever an animal was found to be positive as a result of the detection of GPIC on a vaginal scraping, that animal was always isolation positive. However, chlamydiae were commonly detected by isolation in the presence of a negative scraping.

Treatment with ATS. ATS was produced by immunizing New Zealand White rabbits intradermally with newborn guinea pig thymocytes as previously described (13). The rabbits were initially injected intradermally with 5×10^7 thymocytes in Freund complete adjuvant and were given similar injections 2 weeks later. The animals were given two intravenous inoculations at 2-week intervals. Antiserum obtained from the rabbits was pooled, inactivated at 56°C for 30 min, and then absorbed four times with 10% washed guinea pig erythrocytes. Normal rabbit serum (NRS) (Hazelton Research Products, Inc., Lenexa, Kans.) was also heat inactivated and determined to be free of anti-guinea pig erythrocyte antibodies by hemagglutination. The sera were filter sterilized through 0.45-μm-pore-size filters.

Guinea pigs were injected daily with 1 ml of either ATS or NRS beginning 1 day prior to reinfection with GPIC. The treatment was continued for the duration of the experiment. The effectiveness of ATS treatment was assessed in all animals by sensitization and subsequent challenge with oxazolone and on selected groups by the proliferative response of peripheral blood mononuclear cells to GPIC antigen and concanavalin A (ConA).

Determination of antibody levels. Sera and genital secretions were obtained from guinea pigs as previously described (12, 16) and stored at −20°C until all specimens in the experiment were collected. Serum immunoglobulin G (IgG) was measured by an enzyme-linked immunosorbent assay using HeLa-grown GPIC elementary bodies as antigen and peroxidase-labeled rabbit anti-guinea pig IgG (heavy and light chain specific) (ICN ImmunoBiologicals, Lisle, Ill.) (14). IgA in genital secretions was determined by a similar assay, except that rabbit anti-guinea pig IgA (α chain specific) (ICN) was used, followed by peroxidase-labeled goat anti-rabbit IgG (heavy and light chain specific) (ICN) (14).

Immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretic transfer of chlamydial proteins to nitrocellulose membranes, and immunoblotting assays were performed as previously described (2). Blocking of nitrocellulose membranes was accomplished by using a solution of 150 mM NaCl–10 mM Tris–0.5% (wt/vol) nonfat dry milk (Carnation), pH 7.4. Guinea pig serum specimens were diluted 1:500 to 1:1,000. Vaginal secretions were diluted 1:40 in assays for IgA. In cases in which a sufficient volume of secretion was not available, the entire sample was used for the IgA assay (maximum dilution, 1:80). Localization of antibody binding was accomplished by using rabbit anti-guinea pig IgG (heavy and light chain specific) or IgA (α chain specific) (Miles Laboratories, Inc., Naperville, Ill.), followed by radioiodinated goat anti-rabbit IgG (Organon Teknika, Malvern, Pa.) and autoradiography.

Determination of CMI response. All animals were sensitized with the contact allergen oxazolone (4-ethoxymethylene-2-phenyl oxazolone) about 1 week prior to initiation of ATS and NRS treatment and were tested for their response to challenge 1 week after treatment began. The animals were sensitized on the ear and challenged on a shaved flank as previously described (16). The reactions were assessed on a scale of 0 to 5. Detection of trace erythema was a 1+ response, definite erythema was 2+, deep red erythema was 3+, erythema with edema was 4+, and erythema with tissue necrosis was 5+.

CMI was also assessed on the basis of the proliferative response of peripheral blood mononuclear cells to GPIC antigen and ConA 15 days after initiation of ATS treatment (15). Four milliliters of blood was obtained by cardiac puncture, and the blood was mixed with sodium citrate. The blood was diluted with 3 volumes of RPMI 1640 and centrifuged at $400 \times g$ for 40 min over Histopaque (specific weight, 1.077 g/ml; Sigma Chemical Co., St. Louis, Mo.). Peripheral blood mononuclear cells were collected from the interface, washed, and microcultured at 2×10^5 cells per well in RPMI 1640 containing 10% fetal calf serum, 50 nM 2-mercaptoethanol, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml). UV-inactivated GPIC antigen (7) was added to triplicate wells at 16 μg/ml, and ConA (Sigma) was similarly added at 2.5 μg/ml. Cultures were labeled with [^{3}H]thymidine (1 μCi/ml) during the final 24 h of a 5-day incubation at 37°C in 5% CO_2. The results were expressed as the mean counts per minute of cultures from five animals. The response to GPIC has been found to be dependent upon T cells; B cells make no contribution (L. S. F. Soderberg and R. G. Rank, unpublished data).

Histopathology. At necropsy, the entire genital tract of female guinea pigs was removed and fixed in 10% neutral-buffered Formalin. Sections of the exocervix, endocervix, uterine fundus, bilateral uterine cornua, oviducts, ovaries, and mesosalpinx were stained with hematoxylin and eosin. Each anatomic site was scored for the presence of acute inflammation, plasma cells, lymphocytic aggregates, fibrosis, and, if mucosa was present, mucosal erosion. Sites were scored as follows: trace of parameter, +0.5; presence of the parameter, +1; presence of the parameter at 1 to 4 foci, +2; presence of parameter at more than 4 foci, +3; and confluent presence of the parameter, +4.

Experimental design. To determine the role of CMI in the immune response to a challenge infection, animals were treated with either ATS or NRS 1 day prior to a challenge infection at either day 30 or day 75 after primary infection. These time points were chosen because previous studies have shown that animals are strongly resistant to challenge infection at 30 days, as evidenced by a low infection rate. In contrast, at 75 days, all animals generally become reinfected, although the infection is decreased in intensity and markedly shortened in duration (15). Two separate experiments were performed. In the first experiment, five guinea pigs were each treated with ATS or NRS or remained untreated (NT) and challenged at day 30. Five additional animals in each group were challenged on day 75. In the second experiment, five animals were each treated with ATS or NRS prior to the day 30 challenge and five and four animals were treated with ATS and NRS, respectively, prior to the day 75 challenge. Both experiments were terminated at day 15 to conserve antiserum.

RESULTS

Course of challenge infection. At 30 days after the primary infection, 4 of 10 animals became reinfected in the NRS-

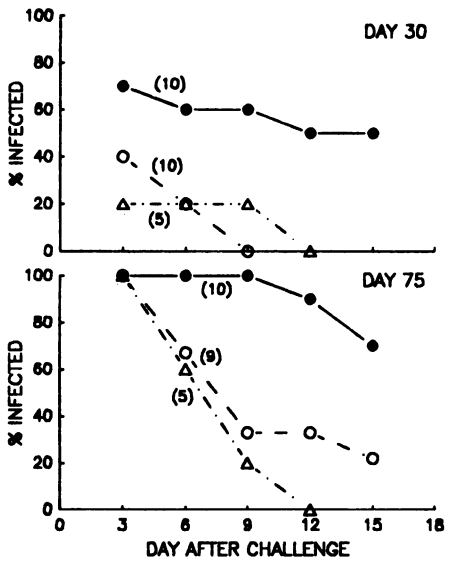

FIG. 1. Course of genital tract infection in guinea pigs challenged either 30 or 75 days after a primary infection. The figure represents the summation of two separate experiments. Guinea pigs were treated with ATS (●) or NRS (○) or were untreated (△). Numbers in parentheses indicate the total number of animals in each group.

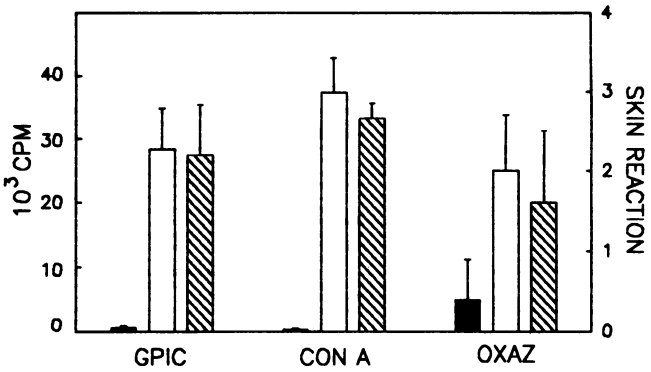

FIG. 2. CMI response in animals treated with ATS (■) or NRS (□) and in animals remaining untreated (▨). The lymphocyte proliferative response to inactivated GPIC antigen or ConA is represented in the first two groups of bars. The skin response to challenge with oxazolone is shown in the last group. The error bars in each case represent the standard deviation.

treated group and 1 of 5 animals became reinfected in the NT group, indicating a high level of immunity at this time (Fig. 1). A slightly higher number of ATS-treated animals (7 of 10) became reinfected, although the difference was not significant when assessed by a Fisher's exact t test. Of the animals which did become reinfected, all of the control animals resolved their infections by day 12 while 5 of 7 of the ATS-treated animals were unable to resolve their infections by the time the experiment was terminated (day 15). The course of the infection in the ATS-treated group, as judged by the percentage of animals remaining infected over time, was significantly different from that in the NRS-treated group when compared by the Wilcoxon signed ranks test ($P < 0.02$). Even though the infections in the ATS-treated group were generally persistent, the level of infection, as demonstrated by the inability to consistently detect organisms on Giemsa-stained vaginal scrapings and by the variability in detection of organisms by isolation, suggested a marked degree of protective immunity. Reinfection in animals in the NRS-treated and NT groups was also detectable only by isolation, not by vaginal scrapings. In a primary infection, chlamydial inclusions are readily detectable on vaginal scrapings for 15 to 20 days.

When guinea pigs were challenged 75 days after the primary infection, all animals became reinfected, regardless of the treatment group (Fig. 1). Once again, most of the ATS-treated guinea pigs remained infected for the course of the infection (7 of 10), whereas most of the animals in the control groups resolved their infections by day 12 (12 of 14). The course of infection in the ATS-treated group was again significantly different from that in the NRS-treated group ($P < 0.04$). The level of infection in all groups was consistently low, as evidenced by negative findings on stained vaginal scrapings.

CMI. To define the effects of ATS treatment on guinea pig immune reactivity, animals were assessed for specific (GPIC) and nonspecific (ConA) blastogenesis and for delayed-type hypersensitivity to oxazolone. The results of these assays from the animals challenged on day 30 are shown in Fig. 2 and are representative of the reactions of all corresponding animals in the two experiments. ATS-treated guinea pigs routinely had a profound deficiency in both parameters of CMI tested, whereas NRS-treated and NT animals had normal responses. The delayed-type hypersensitivity responses of the ATS-treated animals were found to be significantly different from those in the NRS-treated and NT groups by the Mann-Whitney U test ($P < 0.05$). Proliferative responses of ATS-treated animals were also significantly lower than those in either of the control groups when assessed by a one-tailed t test ($P < 0.005$ for both GPIC antigen and ConA).

Humoral immune response. The antibody levels in serum and in genital secretions resulting from GPIC were also measured immediately prior to treatment and reinfection and then were measured again 14 days later to ascertain whether the prolongation of the secondary infection in the ATS-treated group might have been a result of a reduced or absent anamnestic antibody response (Fig. 3). No significant decreases in antibody titers in either serum or secretions were noted in either of the ATS-treated groups (one-tailed t test). The serum IgG titer of the ATS-treated group and the secretion IgA titer of the NT group following the day 75 challenge infection increased significantly ($P < 0.05$ and $P < 0.005$, respectively). There was also a significant decrease in the serum titer of the NRS-treated group after the day 30 challenge. These data indicated that ATS had no obvious effect on the antibody response as measured by enzyme-linked immunosorbent assay .

To evaluate potential effects of ATS on the antigen-specific antibody responses, immunoblot analyses were performed on the prechallenge and day 14 postchallenge sera and genital secretions. Prechallenge sera (data not shown) from animals that were challenged at days 30 and 75 showed IgG responses to, among others, the major outer membrane protein, 61-kilodalton (kDa), 84-kDa, and lipopolysaccharide components that were similar to those we had previously observed (15). The ATS- and NRS-treated groups did not differ in their base-line responses. Likewise, the prechallenge responses in secretions (data not shown) for both IgG and IgA did not differ between the ATS- and NRS-treated groups. The IgA responses were weak and consisted mainly of 61-kDa and lipopolysaccharide responses, while the IgG responses more closely paralleled the serum IgG reactions (15).

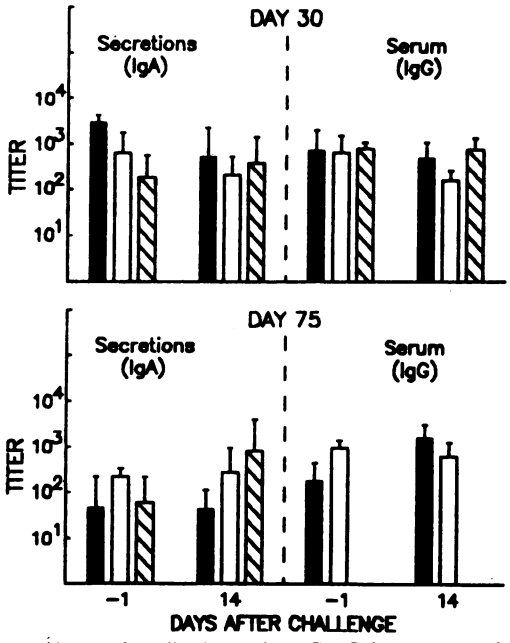

FIG. 3. Titers of antibody against GPIC in serum and genital secretions prior to and 14 days after genital tract challenge in experiment 1. The sample on day −1 was collected before initiation of ATS treatment. Guinea pigs were treated with ATS (■) or NRS (□) or were untreated (▨). The error bars represent the standard deviation.

The secretion IgA response 14 days after challenge infection in both the ATS- and NRS-treated groups for animals in the second experiment are shown in Fig. 4. Reactions to the major outer membrane protein, 61-kDa, and lipopolysaccharide components were observed in each group. Some secretions also contained antibody which bound a 47-kDa protein. Binding appeared more intense in the day 75 group compared with the day 30 group. However, no qualitative differences were observed when ATS- and NRS-treated animals were directly compared within the day 30 challenge group or within the day 75 challenge group. Likewise, IgG binding in serum and in secretions 14 days following challenge was qualitatively very similar when the ATS- and NRS-treated groups were compared (data not shown).

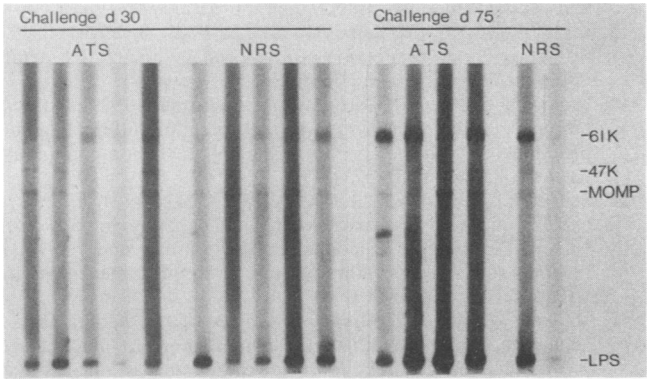

FIG. 4. Autoradiogram showing reactions of vaginal secretions obtained 14 days following a challenge infection in animals treated with rabbit anti-guinea pig thymocyte serum (ATS) or normal rabbit serum (NRS). The secretions are divided according to whether the challenge infection was induced 30 days (challenge d 30) or 75 days (challenge d 75) following an initial infection with GPIC. Immunoreactive bands, lipopolysaccharide (LPS), the major outer membrane protein (MOMP), and 47- and 61-kDa proteins are identified on the right.

Histopathology. Finally, since the data indicated that a deficiency in CMI resulted in prolonged infection upon challenge of immune animals, we examined the genital tracts of four ATS-treated and four NRS-treated animals by histopathology 7 days following a challenge inoculation. The animals had been infected 150 days before. We have previously observed that a large number of mononuclear cells is commonly found in the submucosa of the cervix concomitant with resolution of the infection (1). Thus, it was of interest to determine whether the ATS treatment prevented the influx of these cells. The chronic inflammatory response, which is comprised of mononuclear cells, tended to be of lower intensity in the endocervix and in both upper and lower secretions of the endometrium in the ATS-treated animals, but these differences were not significantly different from the results for the NRS-treated group when analyzed by a Mann-Whitney *U* test (data not shown). Similarly, no differences between the groups were noted in the acute inflammatory response in any of the sites examined.

DISCUSSION

The data presented in this study indicate that when guinea pigs which have recovered from a primary chlamydial genital tract infection become reinfected as a result of a challenge infection, an intact CMI response is required for the resolution of that challenge infection, even in the presence of high titers of specific antibody. Of 17 ATS-treated animals which became reinfected, 12 were unable to resolve the infection in the time frame of the experiment, compared with only 2 of 19 control animals which did not resolve the infection in the same period of time. These results are similar to those seen when guinea pigs were treated with ATS before and during a primary infection with GPIC (13). We have observed previously that animals are more resistant to reinfection when challenged within 1 week of resolution of the primary infection but that virtually all become reinfected when challenged at 8 weeks or more after resolution (15). In the present study it is interesting that resistance to reinfection in the immunologically intact animals was likewise greater at 30 days after infection than at 75 days, but ATS treatment did not seem to alter susceptibility to reinfection significantly, suggesting that CMI may not be the key factor in preventing reinfection soon after resolution of the primary infection.

The importance of CMI in chlamydial genital infections is supported by studies in the B-cell-deficient mouse infected with the agent of mouse pneumonitis, a *C. trachomatis* biovar (11). These mice, which lack humoral immunity but possess CMI, are able to resolve chlamydial genital infections and are immune to reinfection. While the mice fare well in the absence of antibody, a protective function of antibody has not been ruled out in that model.

The prolongation of the infections in ATS-treated guinea pigs was apparently not due to any alteration in the T-cell-dependent antibody response, since antibody could be measured in serum and in secretions and did not differ in level or kinetics compared with controls. Moreover, in the present study, there were no detectable differences in the serum and secretion antibody responses of ATS-treated and control animals with respect to the specific chlamydial antigens recognized. While one would expect some effect on the antibody response by ATS, antibody responses were

well established prior to ATS treatment. In an earlier study, we observed that ATS treatment was even unable to inhibit the production of a primary antibody response in serum or secretions (13). This is consistent with studies involving the immunization of ATS-treated guinea pigs with sheep erythrocytes, in which ATS did not interfere with a T-cell-dependent primary antibody response (17). It is not known why antibody responses are not susceptible to ATS treatment in the guinea pig.

An important point in the present study is that ATS-treated animals, despite their inability to completely resolve the challenge infection, did display a marked degree of immunity, as evidenced by the absence of detectable organisms on stained vaginal scrapings. This immunity might be attributable to the presence of antibody in genital secretions. In an earlier study, when antibody-deficient but CMI-sufficient animals were challenged 28 days after a primary infection, the course of the secondary infection resembled that of the primary infection in intensity (12). Thus, it would appear that antibody is able to diminish the number of organisms but is unable to completely eliminate them.

While we did not examine tissues by histopathology prior to challenge infection, we did study tissues from animals 7 days after the challenge infection to determine whether ATS affected the degree of mononuclear infiltration of the genital tract. While the mononuclear infiltrates in the genital tract were somewhat decreased in ATS-treated animals 1 week after challenge, a strong association between this phenomenon and extended infection cannot be drawn at this point. We have previously demonstrated that the appearance of a mononuclear population in the submucosa occurs at about the time that the infection begins to decrease in intensity (1).

While these studies demonstrate that CMI is necessary to resolve a challenge infection in animals having previously recovered from a genital tract infection, the mechanism by which this resolution occurs is still not clear. It is unlikely that cytotoxic lymphocytes play a major role since they have not been documented to have activity against chlamydia-infected cells (9, 10). Similarly, clearance of organisms by activated macrophages can be ruled out on the basis of the general lack of macrophages in contact with chlamydiae on the genital mucosal surface (1). A more likely possibility is that gamma interferon or other cytokines produced by T cells are able to inhibit the growth of chlamydiae intracellularly or to destroy infected cells, as has been demonstrated in vitro (4, 5). More recently, depletion of gamma interferon by treatment of mice with specific anti-murine gamma interferon antibody has resulted in enhanced respiratory infection with the mouse pneumonitis agent (18). Whether this mechanism also occurs in genital tract infections remains to be determined.

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