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Tumor Necrosis Factor Alpha Activity in Genital Tract Secretions of Guinea Pigs Infected with Chlamydiae

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Previous studies using the guinea pig model of chlamydial genital infection demonstrated that primary infection is associated with a marked acute inflammatory response early on, while chronic inflammation appears later, at a time when the level of infection is reduced. Challenge infections result primarily in a chronic inflammatory response. The stimuli that initiate inflammation and lead to tissue damage have not been defined. We investigated the possibility that tumor necrosis factors (TNFs) play a role in the inflammatory response to chlamydial genital tract infection. Cytotoxicity assays for TNF were performed on genital tract secretions collected from female guinea pigs during infection with the *Chlamydia psittaci* agent of guinea pig inclusion conjunctivitis. During the early days of primary infection, high levels of TNF-α were detected in genital tract secretions from inbred S2 strain and outbred Hartley strain guinea pigs. Significantly lower levels of TNF-α were detected in secretions from both strains during challenge infection. In general, the intensity of the TNF-α response was proportional to the intensity of infection. High TNF-α levels were present during primary infection at a time of marked neutrophil influx. Thus, TNF-α may play an important role in the response to primary chlamydial genital tract infection.

Female genital tract infections with *Chlamydia trachomatis* are primarily asymptomatic in the short term, and yet they are a major cause of chronic infertility and ectopic pregnancy. Because of the limited availability of upper genital tract specimens from humans, definitive information regarding the pathogenesis of pelvic organ disease due to chlamydiae has been difficult to obtain. We have previously characterized the microbiological and pathological effects resulting from vaginal inoculation of female guinea pigs with the chlamydial guinea pig inclusion conjunctivitis agent (GPIC) (1, 19). Histopathology reveals that primary infection is characterized by an acute inflammatory response with a marked influx of polymorphonuclear neutrophils (PMNs), especially early in infection (1, 19). Chronic inflammatory cells such as lymphocytes and plasma cells also participate in the primary immune response, but they appear later, concomitant with the resolution of infection. This predominance of an acute inflammatory response has been observed in other animal models of primary chlamydial infection (16, 17, 23). Of special note is the occurrence of tubal dilatation in some animals after primary infection. Thus, at least one mechanism of tubal damage would appear to involve the acute inflammatory process resulting from an initial chlamydial insult. Challenge infections with GPIC result in a chronic inflammatory infiltrate consisting of lymphocytes and plasma cells (20). A delayed-type hypersensitivity response to chlamydial antigens is thought to be important in the development of chlamydial disease, especially with chronic or repeated infections.

Tumor necrosis factor alpha (TNF-α) and lymphotoxin are proinflammatory cytokines released primarily from monocytes/macrophages and lymphocytes, respectively, upon host invasion by a wide variety of pathogens. These mediators frequently stimulate a cascade of leukocyte-cytokine responses. Thus, they could be important mediators of the host defense against chlamydiae. Further, their abilities to influence fibroblast growth (6) and collagenase release (5) make them potential effectors of chlamydia-induced tissue pathology. In this study, we utilized the guinea pig-GPIC model to investigate the possibility that TNFs play a role in the inflammatory response associated with chlamydial infection of the genital tract.

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MATERIALS AND METHODS

Animals. Female, 20-week-old inbred S2 strain (Robert Koch Institute, Berlin, Germany) or outbred Hartley strain (Sasco Labs, Omaha, Nebr.) guinea pigs were used for experiments. Guinea pigs were given food and water ad libitum in an environmentally controlled room with a cycle of 12 h of light and 12 h of darkness.

Infection. Animals were infected with approximately 10^7 inclusion-forming units of McCoy cell-grown GPIC elementary bodies (EBs) in 0.05 ml via intravaginal inoculation. Control groups received 0.05 ml of medium from mock-infected McCoy cell monolayers. The course of infection was monitored by determination of the percentages of inclusion-bearing cells on Giemsa-stained smears of vaginal wall scrapings obtained at intervals throughout the infection (1). In a single experiment, the isolation of GPIC from cervical swabs was used to confirm infection (18). A group of S2 guinea pigs were given challenge infections with 10^7 inclusion-forming units of GPIC EBs 5 months after their primary infections had resolved. A group of Hartley strain guinea pigs were challenged with GPIC 7 months after primary infection. Vaginal scrapings were done to monitor the course of the challenge infections. All vaginal inoculations were performed at random with respect to the animals' estrus cycles.

Collection of genital tract tissues. In an initial experiment, animals were euthanized by lethal injection of 10% pentobarbital sodium (Nembutal; Abbott Laboratories, Chicago, Ill.), and each genital tract was removed and placed in a petri dish under sterile conditions. The genital tract was cross-sectionally divided immediately proximal to the endocervix, and the oviducts were separated from the uterine horns. The oviductal, uterine, and cervical tissues from each animal were weighed and placed in Eagle minimal essential medium. Volumes which resulted in a concentration of 0.33 g of tissue per ml of medium were used. The tissues were minced under sterile conditions. The tissue suspensions were centrifuged at 300 × *g* for 15 min, and the resulting supernatants were harvested.

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These supernatants were centrifuged again to remove cells and cell debris and then frozen at −70°C until they were used for TNF assays. The tissues were then resuspended in Eagle minimal essential medium with 10% fetal calf serum and 1 μg of gentamicin per ml combined with 5 μg of UV-inactivated GPIC EBs per ml. Volumes which resulted in a concentration of 0.5 g of tissue per ml of medium were used. After an overnight incubation in medium with UV-inactivated EBs, the tissue suspensions were centrifuged twice as described above, and the cell-free supernatants were harvested for TNF assays.

Collection of genital tract secretions. To determine the kinetics of TNF secretion, genital tract secretions were collected from guinea pigs on multiple days throughout the course of primary and challenge GPIC infections. Secretions were collected on days other than those when vaginal scrapings were collected. At intervals before and after infection, an aseptic surgical sponge (2 by 10 mm) (Weck Ophthalmologicals, Atlanta, Ga.) was inserted into the vagina of an anesthetized animal and retrieved 2 h later. The sponges were held at −70°C until they were eluted in 0.5 ml of Eagle minimal essential medium, and the TNF activity of cell-free eluates was measured by the L929 cytotoxicity assay.

Measurement of TNF activity by biological assay. Although immunological reagents for the detection of TNF-α in guinea pigs are not commercially available, interspecies cross-reactivity has been demonstrated for TNF-α receptor-binding activity, and the NH_2-terminal amino acid sequence of guinea pig TNF-α is 76 and 71% homologous to mouse and human TNF-α, respectively. TNF activity was determined by using an adaptation of the lytic assay of Carswell et al. (2) as described previously (4). Briefly, mouse L929 fibroblast cells were plated in 96-well plates at a concentration of 4×10^4 cells per well. After an overnight incubation, supernatants were added to the plates and serially diluted. To determine the specificity of the assay, appropriate dilutions of selected supernatants or standard human recombinant TNF-α (Genentech, San Francisco, Calif.) were preincubated with 5 neutralizing units of polyclonal rabbit anti-human TNF-α (Genentech) or nonspecific rabbit immunoglobulin G for 4 h at 4°C before addition to the plates. Actinomycin D (Sigma, St. Louis, Mo.) was added to each well, and the plates were incubated overnight. Samples were decanted from the adherent L929 cells, and monolayers were washed twice with phosphate-buffered saline (PBS). Residual cells were stained with 0.05% crystal violet in 20% ethanol for 10 min, washed, dried, and eluted with 100 μl of methanol per well. The optical density at 570 nm was determined in a microplate reader. The optical density of L929 cells incubated with medium alone represents 0% lysis, and that of cells treated with 3 M guanidine hydrochloride represents 100% lysis. One unit of TNF is defined as the amount of TNF required to produce 50% lysis. The lower limit of detectability of the assay is 2.5 U/ml.

Immunoblot of guinea pig TNF-α. Proteins in genital tract secretions from guinea pigs infected with GPIC were electrophoresed on 10% acrylamide minigels by the discontinuous denaturing method of Laemmli (12). Before electrophoresis, samples were mixed with an equal volume of solubilizing solution (0.1 M Tris-HCl, pH 6.8) containing 2.5% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.01% bromphenol blue, with or without 5% 2-mercaptoethanol, and boiled for 10 min. Polyacrylamide gel electrophoresis in Tris-glycine buffer (pH 8.6) containing 0.1% SDS was carried out at a constant current of 20 mA. The proteins were electrophoretically transferred to nitrocellulose paper (Millipore, Bedford, Mass.) at 150 mA for 2 h as described by Towbin et al. (26). After blocking, the sheet was incubated overnight at 4°C with a 1:100 dilution of polyclonal rabbit anti-human TNF-α (Genzyme). After washing in PBS, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G diluted 1:1,000 with gelatin diluent. Blots were developed with alkaline phosphatase color developer for 10 min. Human recombinant TNF-α monomer (Genentech) was used as a positive control, and guinea pig secretions with low levels of TNF-α as determined by biological assay were used as negative controls.

Statistics. Statistical comparisons between groups were made by a two-factor (either days and status of infection or days and strain) analysis of variance with repeated measures (RM ANOVA) on one factor (days). Comparisons within groups were made by using the Student-Newman-Keuls method of all-pairwise multiple comparisons with Sigma Stat statistical software (Jandel Scientific, Erkrath, Germany). Correlations were determined by the Spearman rank order correlation method (Sigma Stat; Jandel Scientific).

RESULTS

TNF activity in genital tract tissues of infected and uninfected guinea pigs. In initial experiments we sought to determine TNF activity levels from infected tissues directly. Little or no TNF activity was detected in supernatants harvested directly from minced tissues taken from S2 animals 5 or 9 days after vaginal inoculation (Table 1). This was true for supernatants from both infected and uninfected animal tissues. Overnight incubation of uninfected tissues with 5 μg of UV-inactivated EBs per ml also did not induce TNF release. However, when genital tract tissues from infected animals sacrificed on day 5 or 9 were incubated overnight with UV-inactivated EBs, TNF activity in the resulting supernatants was successfully measured (Table 1). The TNF activity detected by the L929 assay was neutralized 95 to 100% by preincubation of the supernatants with polyclonal rabbit anti-human TNF-α. Thus, although little or no TNF was detected from the infected tissues directly, these data suggest that prior infection with chlamydiae primes the tissue for a TNF-α response upon subsequent exposure to chlamydial proteins.

TABLE 1. TNF-α in uninfected and infected guinea pig tissues: modulation by overnight incubation with UV-inactivated GPIC EBs

Day	Tissue	TNF-α (U/ml/g of tissue)[a]	
		Pre-EBs	Post-EBs
5	Uninfected		
	Ovary	<10	20 ± 10
	Uterus	<10	10 ± 10
	Cervix	<10	10 ± 10
	Infected		
	Ovary	<10	290 ± 70
	Uterus	<10	30 ± 10
	Cervix	40 ± 40	1,150 ± 670
9	Uninfected		
	Ovary	<10	<10
	Uterus	<10	<10
	Cervix	<10	<10
	Infected		
	Ovary	<10	60 ± 30
	Uterus	<10	40 ± 10
	Cervix	<10	440 ± 170

[a] Expressed as means ± SEM for triplicate determinations with three uninfected and four infected animals 5 or 9 days after inoculation. Anti-TNF-α neutralized cytotoxicity 100%. Pre- and post-EBs, before and after incubation with inactivated GPIC EBs, respectively.

We knew that the detection of TNF from tissues would be difficult. We hoped to increase the sensitivity of detection by measuring TNF activity levels in genital tract secretions collected via vaginal sponges. We felt that the cytokine would be relatively protected from tissue proteases once adsorbed onto a sponge and would be taken up less by receptors and binding proteins present in the tissues. Indeed, the results of L929 assays performed on the genital tract secretions collected from these animals were markedly different from those for tissues. The mean TNF activities in genital tract secretions from uninfected guinea pigs collected 5 and 9 days after vaginal inoculation of buffer were 4.6×10^3 and 2.1×10^3 U/ml, respectively. In contrast, the secretions obtained from guinea pigs 5 days after inoculation of GPIC contained extremely high levels of TNF (mean activity = 19.2×10^3 U/ml). The secretions obtained on day 9 of infection yielded a mean TNF-α level of only 2.4×10^3 U/ml. Cervical isolations were positive for all of the infected animals and none of the uninfected animals (data not shown). The TNF activity detected by the L929 assay was neutralized >95% by preincubation of the supernatants with polyclonal anti-human TNF-α. Nonspecific rabbit immunoglobulin G did not affect TNF-induced L929 cytotoxicity. Although the number of animals tested was small, these data suggest that chlamydial infection induced a significant TNF-α response in the mucosa of the genital tract. In addition, we had developed a sensitive method of quantifying this response over time.

Immunoblotting of guinea pig TNF-α. For further confirmation that the cytokine we were detecting was indeed TNF-α, we performed immunoblots as described in Materials and Methods. Proteins in secretions from inbred S2 strain guinea pigs were separated on denaturing gels and then transferred and

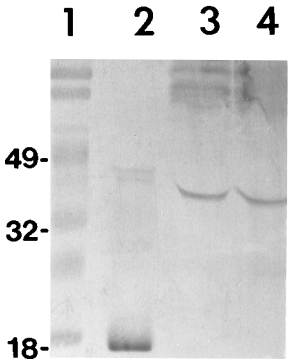

FIG. 1. Immunoblot of S2 guinea pig cervical secretions after primary infection with GPIC. Proteins were electrophoresed on a nonreducing denaturing gel, transferred to nitrocellulose, and probed with a polyclonal rabbit antibody to human TNF-α. Lane 1, prestained molecular mass markers; lane 2, recombinant 17-kDa monomer of human TNF-α. Monomers which have reannealed to the 40-kDa trimer form of TNF-α are evident in lane 2. Lanes 3 and 4, secretions eluted from genital tract sponges obtained from S2 animals on days 3 and 5, respectively, of primary infection. Molecular masses (in kilodaltons) are shown on the left.

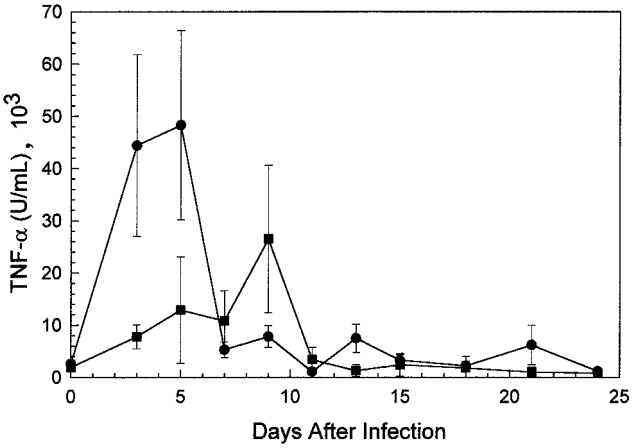

FIG. 3. Mean (± SEM) TNF-α levels in genital tract secretions of inbred S2 strain guinea pigs ($n = 5$) over the courses of primary (circles) and challenge (squares) infections with 10^7 inclusion-forming units of GPIC. Cytotoxicity assays with L929 fibroblasts were used to detect TNF. All specimens were run in duplicate. Preincubation of secretions with anti-human TNF-α antibody resulted in >95% neutralization of activity in every instance.

probed with polyclonal anti-human TNF-α antibody. The 40-kDa trimer of TNF-α was detected in secretions collected on days 3 and 5 of primary infection (Fig. 1, lanes 3 and 4, respectively). The active form of TNF-α in vivo is a 40-kDa trimer. A faint band at approximately 40 kDa can also be seen in Fig. 2, lane 2, representing recombinant human TNF-α monomers that reannealed in vitro. We were not able to denature the TNF-α in secretions unless we added 2-mercaptoethanol to the solubilizing solution. When secretions from primary infections in inbred S2 strain and outbred Hartley strain guinea pigs were solubilized with the addition of 2-mercaptoethanol, the 17-kDa monomer form of TNF-α was detected in addition to the 40-kDa trimer (Fig. 2). Bands were absent from lanes representing secretions containing baseline levels of TNF-α as detected by biological assay (data not shown). Guinea pig TNF-α has >70% homology with human TNF-α and has the same molecular weight (24).

TNF activity in female guinea pig genital tract secretions during primary and challenge infections with GPIC. Secretions collected from five inbred S2 strain female guinea pigs 1 day prior to vaginal inoculation with GPIC EBs and on multiple days during the courses of both primary and challenge infections were assayed for TNF (Fig. 3). Extremely high levels of TNF were detected in all five animals on days 3 to 5 of primary infection, with four of five guinea pigs having levels greater than 40×10^3 U/ml. Secretions taken on day 7 yielded TNF activity levels near the baseline level. Although moderate amounts of TNF were detected in some of the animals during the second 2 weeks of primary infection, the levels did not approach those seen earlier in the infection. Levels determined through day 19 were generally low, although individual animals had levels as high as 9.6×10^3, 11.1×10^3, 15.4×10^3, 17.9×10^3, and 20.2×10^3 U/ml. Preincubation of supernatants in anti-TNF-α antibody resulted in >95% neutralization of TNF activity in every instance (data not shown). Thus, a burst of TNF-α activity was detected in the genital tract mucosal responses of S2 strain guinea pigs upon primary infection with GPIC.

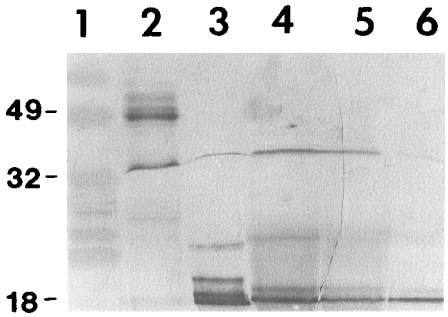

FIG. 2. Immunoblot of S2 and Hartley strain guinea pig cervical secretions after primary infection with GPIC. Proteins were electrophoresed on a denaturing reducing gel, transferred to nitrocellulose, and probed with a polyclonal rabbit antibody to human TNF-α. Lanes 1 and 2, prestained molecular mass markers; lanes 3 and 4, cervical secretions from S2 animals 3 and 5 days, respectively, after primary infection; lane 5, cervical secretions from Hartley strain guinea pigs 5 days after infection; lane 6, recombinant monomer of human TNF-α. Molecular masses (in kilodaltons) are shown on the left.

Five months after resolution of their first infection, the S2 strain guinea pigs were challenged with GPIC and secretions were collected for TNF determinations, (Fig. 3). Practically all of the TNF activity detected was again due to TNF-α, as preincubation of eluted secretions with anti-TNF-α antibody resulted in >95% neutralization of cytotoxicity in every instance (data not shown). Levels of TNF detected 1 day prior to challenge were relatively low (range of 0.3×10^3 to 5.8×10^3 U/ml). The overall TNF-α response after challenge infection was significantly lower ($P = 0.008$) than that after primary infection when the responses were compared by two-way RM ANOVA. Relative increases in TNF-α activity were detected in four of five animals on at least one or two occasions during challenge infection. The day of the peak response varied among individual animals; one peak was on day 3 (15.4×10^3 U/ml), one was on day 5 (53.7×10^3 U/ml), and two were on day 9 (90.1×10^3 and 16.3×10^3 U/ml, respectively). The peak response as determined by calculation of the mean was seen on day 9. By day 11 of challenge infection, the TNF-α activity levels were back to the baseline level in all animals (Fig. 3).

The courses of primary and challenge infections in inbred S2 strain guinea pigs after vaginal inoculation with GPIC EBs are depicted in Fig. 4. During primary infection, all animals were positive on days 4 to 18, with peak numbers of organisms found

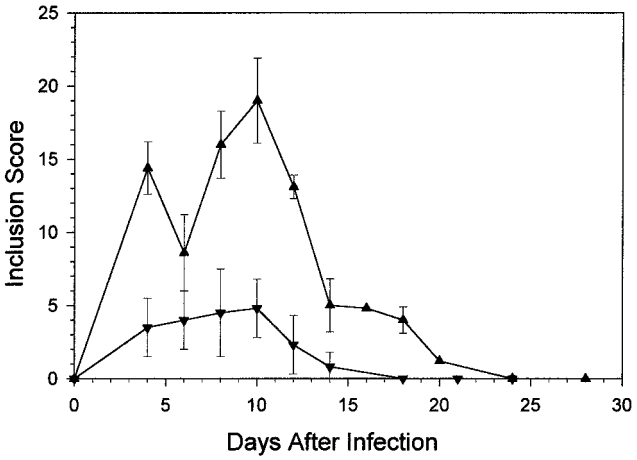

FIG. 4. Courses of primary (▲) and challenge (▼) infections with GPIC in inbred S2 strain guinea pigs. The level of infection was determined by calculation of the percentages of inclusion-bearing cells in Giemsa-stained smears of vaginal wall scrapings. Data points represent means ± SEM for duplicate determinations with five animals on each day examined.

on days 8 to 10. Primary infections had declined by day 12. Most animals resolved their initial infections by day 20, and all scrapings were negative for organisms by days 24 to 28 (Fig. 4).

Although all five S2 animals became infected upon challenge, positive evidence of infection was delayed in one animal until day 6 and in another until day 8. All animals were negative by day 15 (Fig. 4). As previously seen, the course of challenge infection was significantly attenuated ($P < 0.0005$) compared with the course of primary infection when inclusion scores were compared by two-way RM ANOVA (20).

A comparison of mean TNF-α levels and inclusion scores during primary infection reveals that mucosal TNF-α responses peaked several days prior to the peak in numbers of inclusions (Fig. 3 and 4). Infected animals exhibited some evidence of increased TNF-α activity intermittently throughout the course of the primary infection. However, as the majority of vaginal scrapings became negative for inclusions, TNF-α activity decreased to minimum levels (Fig. 3 and 4). There was a positive correlation (correlation coefficient = 0.38; $P = 0.006$) between TNF-α and inclusion scores during primary infection.

A comparison of TNF-α levels and inclusion scores during challenge infection reveals that both parameters underwent slow, moderate increases through day 10 (Fig. 3 and 4). Mean TNF-α levels peaked at around the same time as the mean peak in inclusion scores. As the challenge infection waned, TNF-α activity dropped to the baseline level. As with the primary infection, there was a positive correlation ($P = 0.03$; correlation coefficient = 0.31) between TNF-α levels and inclusion scores during challenge infection.

TNF activity in outbred female Hartley strain guinea pigs during primary and challenge infections with GPIC. We repeated the primary and challenge infection experiments with five outbred Hartley strain female guinea pigs. Practically all of the TNF activity detected during both primary and challenge infections was TNF-α, as evidenced by >95% neutralization of L929 cytotoxicity with anti-TNF-α (data not shown). Moderate increases in TNF-α activity were detected in the Hartley animals during the course of primary infection with GPIC (Fig. 5). However, only two of five animals had levels of TNF-α activity that were greater than 30×10^3 U/ml. The highest-level responses were detected on day 5 of primary infection, when one animal had a mean response of 88.3×10^3 U/ml and a second had a mean level of 47.7×10^3 U/ml. One animal exhibited a delayed response, with significant TNF-α activity (30.1×10^3 U/ml) not seen until day 9. In another animal, 6.1×10^3 U/ml was the highest level of TNF-α detected, and this peak did not occur until day 7. Thus, significant variability was seen among individual Hartley animals in the amount of TNF-α detected during primary infection as well as in the day of the peak TNF-α response. Moderate levels of TNF-α activity were detected intermittently during the second 2 weeks of primary infection, with levels nearing the baseline level after day 21 (Fig. 5).

When these guinea pigs were reinoculated with GPIC 7 months after resolution of their primary infections, TNF-α activity did not increase above the baseline level. Prior to challenge, three animals had TNF-α activity levels of approximately 4.5×10^3 U/ml, resulting in a relatively high mean baseline TNF-α level of 2.6×10^3 U/ml (Fig. 5). When the means ± standard errors of the mean (SEM) for TNF-α levels detected over the course of challenge infection were compared with those for TNF-α levels during primary infection by a two-way RM ANOVA, a significant difference was determined ($P = 0.04$). Thus, in the outbred Hartley strain, primary infection induced a significantly greater TNF-α response than challenge infection (Fig. 5).

The duration and intensity of challenge infection were also significantly attenuated with respect to those of primary infection ($P < 0.0005$ by two-way RM ANOVA; data not shown). Thus, in both guinea pig strains, primary infections were of significantly greater intensity and induced a significantly greater TNF-α response than challenge infections. As with the S2 strain, there was a direct correlation between inclusion scores and TNF-α levels during primary infection in the Hartley strain animals (correlation coefficient = 0.30; $P < 0.05$). There was no significant relationship between TNF-α levels and inclusion scores during challenge for the Hartley strain ($P > 0.05$). Consideration of the data from both groups indicates that the overall TNF-α response paralleled the level of infection and correlated with the absolute number of organisms in the genital tract epithelium.

Secretions from control Hartley strain guinea pigs inocu-

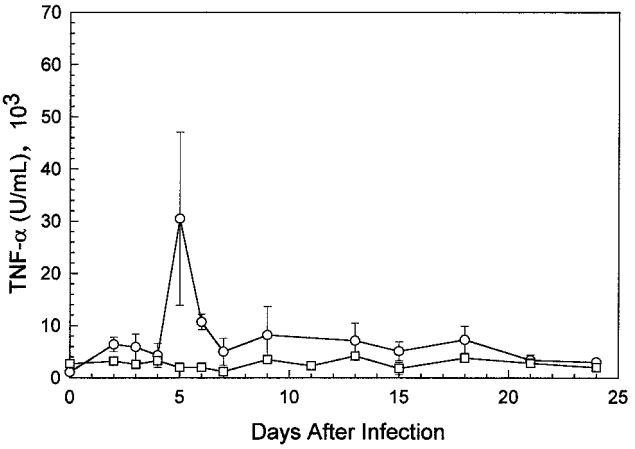

FIG. 5. Mean (± SEM) TNF-α levels in genital tract secretions of outbred Hartley strain female guinea pigs ($n = 5$) over the courses of primary (circles) and challenge (squares) infections with 10^7 inclusion-forming units of GPIC. Cytotoxicity assays with L929 fibroblasts were used to detect TNF. All specimens were run in duplicate. Preincubation of secretions with anti-human TNF-α antibody resulted in >95% neutralization of activity in every instance.

lated with material from mock-infected McCoy cell monolayers yielded baseline levels of TNF-α activity. The largest amount detected in a control animal was 3.2×10^3 U/ml 4 days after mock infection. The highest mean level of TNF-α was 1.5×10^3 U/ml, detected on day 4 of mock infection.

DISCUSSION

TNF-α has frequently been documented as an initiatory cytokine released upon bacterial invasion of the host. It can induce a significant influx of PMNs and has a strong activating effect on these cells in addition to other leukocytes and endothelial cells. A prominent acute inflammatory response is seen upon chlamydial infection of the genital tract (1, 11). The stimulus for this mucosal influx of PMNs has not been determined. Williams et al. (30) demonstrated that TNF-α played a role in the host defense against chlamydiae in a murine model of pneumonia caused by the *C. trachomatis* agent of mouse pneumonitis. In this model, in which mortality is 90% by 10 days after infection, TNF was detected in lung homogenates by day 3. In such an overwhelming infection, it is not surprising that significant levels of this proinflammatory cytokine would be seen.

In initial experiments, we attempted to detect the production of TNF-α directly from genital tract tissues of guinea pigs infected with the *Chlamydia psittaci* GPIC. We were unable to detect TNF directly from infected tissues, but upon subsequent exposure to chlamydial proteins in the form of inactivated EBs, significant levels of TNF-α were detected from these same tissues. These data suggested that TNF might play a role in the inflammatory response associated with a chlamydial infection. Direct demonstration of TNF-α production by specific tissues is often difficult. TNF-α is efficiently secreted from macrophages and PMNs, making immunocytochemical detection generally impossible. In addition, since TNF-α biosynthesis is largely controlled at a translational level (7), detection of TNF-α mRNA may not indicate that synthesis of the protein has occurred.

Because chlamydial infection and the associated inflammatory response remain restricted to the genital tract, we designed a novel method for detecting TNF in genital tract secretions. With two different groups of female guinea pigs, we detected extremely high levels of TNF-α in genital tract secretions collected early during primary infection. Considering the magnitude of the TNF-α response in individual animals on the initial day that they were examined, it is likely that TNF-α secretion began very soon after the animals were inoculated with GPIC. This acute rise in TNF-α levels coincides with an acute increase in the numbers of infected epithelial cells detected in vaginal and endocervical scrapings. Although moderate increases in TNF-α levels were detected during the second 2 weeks of infection in some of the animals in each group, genital tract secretions collected after day 5 of primary infection generally contained small amounts of TNF-α activity relative to those seen early on.

Previous histopathological studies in our laboratory with the GPIC model have revealed that a marked acute inflammatory response, characterized by infiltration of the cervical epithelium with PMNs, is present on day 5 of primary infection (1). It is interesting that the acute inflammatory response appears concomitantly with the increase in TNF-α levels. By days 7 to 9, acute inflammation in the lower genital tract begins to wane, and by day 10, the PMN population is decreased. Paralleling the decrease in PMNs is a corresponding increase in mononuclear cells. By day 14 the inflammatory response has subsided considerably, and histopathology in the lower genital tract is essentially normal by day 20. Thus, these data suggest an association between the appearance of chlamydial inclusions, the onset of the acute inflammatory response, and the production of TNF-α in the lower genital tract during primary infection. It remains to be determined whether TNF-α is associated with the acute inflammatory response in the upper genital tract as well.

The levels of TNF-α detected during primary infection in the Hartley strain guinea pigs were somewhat lower than those detected in the S2 animals, although a direct comparison was not made. The lower TNF-α response was not due to different levels of infection, as vaginal scrapings revealed that the numbers of organisms and durations of infection were similar in these two groups. Strain differences may explain the difference in TNF-α levels, with more consistent increases being seen in the genetically inbred S2 animals than in the outbred Hartley animals.

In contrast to the apparent direct relationship between TNF-α and PMN infiltration during the early primary immune response, when the primary response shifts to a predominance of chronic mononuclear cells, relatively small amounts of TNF-α are detected in genital tract secretions. In addition, secretions collected from guinea pigs during a challenge infection with GPIC contained levels of TNF-α that were significantly lower than those during primary infection. These results may reflect the change in inflammatory response in naive versus previously infected animals. The presence of an anamnestic immune response results in a markedly diminished acute inflammatory reaction and a relatively enhanced chronic or mononuclear inflammatory response (20). It is quite possible that different mechanisms for the release of TNF-α may be active in primary and challenge infections. Moreover, the amount of TNF-α produced may also be affected by the significantly lower intensity of infection in a challenge versus a primary infection.

The relatively rapid burst of TNF-α activity detected upon primary vaginal inoculation of guinea pigs with GPIC suggests that the cell source for this cytokine resides in the genital tract. Macrophages are known to be a major source of the early TNF-α response seen with acute inflammation, and it is likely that there are adequate numbers of macrophages in the genital tract epithelium to account for TNF-α production. Langerhans cells are prevalent in the cervicovaginal epithelia of humans, localized both in the basal part and near the surface of the epithelium (8). Monocytes and tissue macrophages are also routinely detected in transformation zone epithelia and endocervical tissues in humans. Langerhans cells, monocytes, and resident PMNs of the genital tract epithelium, combined with other genital tract cell types, e.g., endothelial cells, mast cells, and fibroblasts, may contribute to the production and release of TNF-α. In an experimental model of gonococcal salpingitis which employs human fallopian tubes in organ culture, gonococcal infection results in mucosal production and release of TNF-α (13). Thus, it is possible that the genital tract epithelial cells themselves may be among the first to release TNF-α. Such TNF-α release could then activate local macrophages and stimulate PMN influx and adherence, promoting further production and release of TNF-α. Since lymphocytes appear later during the response to a primary infection, it seems unlikely that they contribute significantly to this early TNF-α response.

It is quite apparent that in the guinea pig-GPIC model, as in other animal models, major pathology during a primary infection in all portions of the genital tract, including the oviduct, results from an acute inflammatory response which progresses to a chronic mononuclear response and fibrosis (17, 19, 27). However, the stimuli which initiate the acute inflammatory

response and participate in tissue damage have not been defined. Certainly, it is obvious that the inflammatory response requires chlamydial infection before it can be initiated. Thus, the primary stimulus for the initiation of the acute inflammatory response should be the organism itself. Chlamydial EBs bind C3 and thereby initiate the alternative complement pathway (14); the binding site was found to be the chlamydial major outer membrane protein. However, a role for chlamydial lipopolysaccharide (LPS) in the initiation of the alternative pathway cannot be ruled out, especially since LPS is expressed in large amounts on the surfaces of infected cells early in the infection cycle (10, 22). Thus, one possible mechanism of inflammation may be the attraction of PMNs to the site by chemotactic factors associated with the activation of the alternative complement pathway. Indeed, Megran et al. (14) and Register et al. (21) reported that *C. trachomatis* and *C. psittaci* EBs, respectively, were chemotactic for PMNs in the presence of complement.

Alternatively, PMNs may be recruited through the release of TNF-α from PMNs or macrophages as a result of complement activation (15) or LPS stimulation. TNF-α has multiple effects with regard to inflammation and tissue injury, such as being chemotactic for monocytes and PMNs, increasing the expression of adhesion molecules on endothelial cells so that PMN migration can occur, activating PMNs, inducting interleukin-6 (which may in turn induce secretion of transforming growth factor β), stimulating fibroblast growth (6) and collagenase release (5), and many other effects (28). Any or all of these functions could influence the course of chlamydial disease in the genital tract.

While one might expect TNF-α to have a role in the production of tissue damage, only one study has implicated it as having cytotoxic activity for chlamydia-infected cells (29). In the in vitro experimental model of acute gonococcal salpingitis described by McGee et al. (13), recombinant human TNF-α damaged fallopian tube mucosal cells in a dose-dependent manner and produced epithelial damage with the same ultrastructural features as those observed in gonococcal infection. Blocking the production of TNF-α during gonococcal infection diminished the extent of damage to fallopian tube mucosal cells in vitro, suggesting that the pathology induced by *Neisseria gonorrhoeae* is partially mediated by TNF-α. In contrast to in vitro models of gonococcal salpingitis, in which infection results in epithelial cell damage, infection of human fallopian tube organ cultures with *C. trachomatis* produces no direct damage of the mucosal epithelium (3, 9). In chlamydial genital tract disease, immune response cells may be necessary at the site of infection for the induction of a TNF-α response. There is only minimal evidence of an association of TNF-α with salpingitis in vivo. Toth et al. (25) detected the presence of TNF-α in fluids from damaged fallopian tubes in patients with salpingitis but not in fluids from undamaged tubes.

In vitro studies demonstrate that human cultured monocytes/macrophages are highly chlamydiacidal towards ocular-genital tract serovars of chlamydiae (31). If the magnitude of the TNF-α response detected in genital tract secretions reflects the relative chlamydiacidal activity of genital tract monocytes and macrophages, it is possible that increased levels of TNF-α are associated with more effective early eradication of the organism. This would lead to a decreased chance for persistent or chronic infection and thus to a decrease in tissue pathology associated with chronic inflammation. Of course, another possibility is that TNF-α is a "double-edged sword" in this disease as it is in many others. Meticulous fine-tuning of this response may be necessary in order to carry out efficient eradication of the chlamydial organism without causing tissue damage in the process.

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REFERENCES

1. **Barron, A. L., H. J. White, R. G. Rank, and B. L. Soloff.** 1979. Target tissues associated with genital infection of female guinea pigs by the chlamydial agent of guinea pigs inclusion conjunctivitis. J. Infect. Dis. **139:**60–68.
2. **Carswell, E. A., L. J. Old, R. I. Kassell, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72:**3666–3670.
3. **Cooper, M. D., J. Rapp, C. Jeffery-Wiseman, R. C. Barnes, and D. S. Stephens.** 1990. *Chlamydia trachomatis* infection of human fallopian tube organ cultures. J. Gen. Microbiol. **136:**1109–1115.
4. **Darville, T., D. Tabor, K. Simpson, and R. F. Jacobs.** 1994. Intravenous immunoglobulin modulates human mononuclear phagocyte tumor necrosis factor-α production *in vitro*. Pediatr. Res. **35:**397–403.
5. **Dayer, J. M., B. Beutler, and A. Cerami.** 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J. Exp. Med. **162:**2163–2168.
6. **Elias, J. A., R. C. Krol, B. Freundlich, and P. M. Sampson.** 1988. Regulation of human lung fibroblast glycosaminoglycan production by recombinant interferons, tumor necrosis factor and lymphotoxin. J. Clin. Invest. **81:**325–333.
7. **Han, J., T. Brown, and B. Beutler.** 1990. Endotoxin-responsive sequences control cachectin/TNF biosynthesis at the translational level. J. Exp. Med. **171:**465–475.
8. **Hussain, L. A., C. G. Kelly, R. Fellowes, E.-M. Hecht, J. Wilson, M. Chapman, and T. Lehner.** 1992. Expression and gene transcript of Fc receptors for IgG, HLA class II antigens and Langerhans cells in human cervico-vaginal epithelium. Clin. Exp. Immunol. **90:**530–538.
9. **Hutchinson, G. R., D. Taylor-Robinson, and R. R. Dourmashkin.** 1979. Growth and effect of chlamydiae in human and bovine oviduct organ cultures. Br. J. Vener. Dis. **55:**194–202.
10. **Karimi, S. T., R. H. Schloemer, and C. E. Wilde.** 1989. Accumulation of chlamydial lipopolysaccharide antigen in the plasma membranes of infected cells. Infect. Immun. **57:**1780–1785.
11. **Kiviat, N. B., J. A. Paavonen, J. Brockway, C. W. Critchlow, R. C. Brunhan, C. E. Stevens, W. E. Stammm, C. C. Kuo, T. DeRoven, and K. K. Holmes.** 1985. Cytologic manifestations of cervical and vaginal infections. JAMA **253:**989–993.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **McGee, Z. A., C. M. Clemens, R. L. Jensen, J. J. Klein, L. R. Barley, and G. L. Gorby.** 1992. Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. Microb. Pathog. **12:**333–341.
14. **Megran, D. W., H. G. Stiver, and W. R. Bowie.** 1985. Complement activation and stimulation of chemotaxis by *Chlamydia trachomatis*. Infect. Immun. **49:**670–673.
15. **Okusawa, S., K. B. Yancey, J. W. M. van der Meer, S. Endres, G. Lonnemann, K. Hefter, M. M. Frank, J. F. Burke, C. A. Dinarello, and J. A. Gelfund.** 1988. C5a stimulates secretion of tumor necrosis factor from human mononuclear cells *in vitro*. J. Exp. Med. **168:**443–448.
16. **Patton, D. L., S. A. Halbert, C. C. Kuo, S. P. Wang, and K. K. Holmes.** 1983. Host response to primary *Chlamydia trachomatis* infection of the fallopian tube in pig-tailed monkeys. Fertil. Steril. **40:**829–840.
17. **Patton, D. L., D. V. Landers, and J. Schachter.** 1989. Experimental *Chlamydia trachomatis* salpingitis in mice: initial studies on the characterization of the leukocyte response to chlamydial infection. J. Infect. Dis. **159:**1105–1110.
18. **Rank, R. G., B. E. Batteiger, and L. S. F. Soderberg.** 1988. Susceptibility to reinfection after a primary chlamydial genital infection. Infect. Immun. **56:**2243–2249.
19. **Rank, R. G., and M. M. Sanders.** 1992. Pathogenesis of endometritis and salpingitis in a guinea pig model of chlamydial genital infection. Am. J. Pathol. **140:**927–936.
20. **Rank, R. G., M. M. Sanders, and D. L. Patton.** 1995. Increased incidence of oviduct pathology in the guinea pig after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. Sex. Transm. Dis. **22:**48–54.
21. **Register, K. B., P. A. Morgan, and P. B. Wyrick.** 1986. Interaction between *Chlamydia* spp. and human polymorphonuclear leukocytes in vitro. Infect. Immun. **52:**664–670.

22. **Richmond, S. J., and P. Stirling.** 1981. Localization of chlamydial group antigen in McCoy cell monolayers infected with *Chlamydia trachomatis* or *Chlamydia psittaci*. Infect. Immun. **34:**561–570.
23. **Ripa, K. T., B. R. Moller, P. A. Mardh, E. A. Freundt, and F. Melsen.** 1979. Experimental acute salpingitis in grivet monkeys provoked by *Chlamydia trachomatis*. Acta. Pathol. Microbiol. Scand. Sect. B **87:**65–70.
24. **Tamatani, T., S. Kimura, T. Hashimoto, and K. Onozaki.** 1988. Purification of guinea pig tumor necrosis factor (TNF): comparison of its antiproliferative and differentiative activities for myeloid leukemic cell lines with those of recombinant human TNF. J. Biochem. **105:**55–60.
25. **Toth, M., J. Jeremias, W. J. Ledger, and S. S. Witkin.** 1992. *In vivo* tumor necrosis factor production in women with salpingitis. Surg. Gynecol. Obstet. **174:**359–362.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
27. **Tuffrey, M., P. Alexander, C. Woods, and D. Taylor-Robinson.** 1992. Genetic susceptibility to chlamydial salpingitis and subsequent infertility in mice. J. Reprod. Fertil. **95:**31–38.
28. **Vassalli, P.** 1992. The pathophysiology of tumor necrosis factor. Annu. Rev. Immunol. **10:**411–452.
29. **Williams, D. M., L. F. Bonewald, G. D. Roodman, G. I. Byrne, D. M. Magee, and J. Schachter.** 1989. Tumor necrosis factor alpha is a cytotoxin induced by murine *Chlamydia trachomatis* infection. Infect. Immun. **57:**1351–1355.
30. **Williams, D. M., D. M. Magee, L. F. Bonewald, J. G. Smith, C. A. Bleicker, G. I. Byrne, and J. Schachter.** 1990. A role in vivo for tumor necrosis factor alpha in host defense against *Chlamydia trachomatis*. Infect. Immun. **58:** 1572–1576.
31. **Yong, E. C., E. Y. Chi, and C.-C. Kuo.** 1978. Differential antimicrobial activity of human mononuclear phagocytes against human biovars of *Chlamydia trachomatis*. J. Immunol. **139:**1297–1306.

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