An important question in the study of chlamydial genital tract disease is why some women develop severe upper tract disease while others have mild or even “silent” infections with or without pathology. Animal studies suggest that the pathological outcome of an infection is dependent upon both the composition of the infecting chlamydial population and the genotype of the host, along with host physiological effects, such as the cyclical production of reproductive hormones and even the size of the infecting inoculum or the number of repeated infections. In this study, we compared two variants of *Chlamydia caviae*, contrasting in virulence, with respect to their abilities to ascend the guinea pig genital tract. We then determined the effect of combining the two variants on the course of infection and on the bacterial loads of the two variants in the genital tract. Although the variants individually had similar infection kinetics in the cervix, SP6, the virulent variant, could be isolated from the oviducts more often and in greater numbers than the attenuated variant, AZ2. SP6 also elicited higher levels of interleukin 8 (IL-8) in the lower genital tract and increased leukocyte infiltration in the cervix and uterus compared to AZ2. When the two variants were combined in a mixed infection, SP6 outcompeted AZ2 in the lower genital tract; however, AZ2 was able to ascend the genital tract as readily as SP6. These data suggest that the ability of SP6 to elicit an inflammatory response in the lower genital tract facilitates the spread of both variants to the oviducts.

One of the important questions in the study of chlamydial genital tract disease is why some women develop severe upper tract disease while others have either mild or even “silent” infections with or without pathology. There is probably no single all-encompassing explanation, and the answer is likely a combination of factors, including the genital tract physiology and the genetic background of the individual and importantly, the number of repeated infections that an individual may experience. In addition, because the female genital tract undergoes cyclical hormonal changes, the ability of the organism to produce disease at different times during the menstrual cycle may be altered. Sweet and colleagues showed an increased incidence of chlamydial infection associated with infection at distinct times during the menstrual cycle (1). We previously observed that infection of female guinea pigs with *Chlamydia caviae* 7 days prior to estrus resulted in more severe upper tract pathology than in animals infected at estrus or 7 days after estrus (2). There is no doubt that the genetic makeup of the individual can have a major impact on the disease outcome, as well. Several studies demonstrated variability in oviduct pathology based on the strain of mouse infected (3, 4). More recently, Su and coauthors studied the development of oviduct pathology following *Chlamydia muridarum* infection of various strains of a *C57BL*/*6 and *DBA/2* cross and were able to define an interval of 17 genes in a 2.2-Mb region of mouse chromosome 3 that was associated with the severity phenotype (5). They further identified a 3.5-Mb locus on mouse chromosome 19, containing coding sequences for 17 genes, that was associated with a phenotype of increased uterine pathology. Thus, these data clearly demonstrate that some individuals may have a specific genotype making them more susceptible to the development of severe disease.

Finally, it has also become apparent that individuals are infected with a population of multiple chlamydial genetic variants with differing pathological potentials. Sturdevant and colleagues plaque purified multiple isolates from mice infected genitally with *C. trachomatis* and found that the clonal isolates could be defined by the length of infection that they elicited (6). The fact that the isolates were derived from an infection stock strongly suggested that the stock consisted of a population of variants. We recently plaque purified five separate isolates from a mouse infected with the *C. muridarum* Nigg strain and found distinct differences in the pathology phenotype of each variant, ranging from one variant eliciting hydrosalpinx in 20% of oviducts to another variant eliciting hydrosalpinx in 80% of oviducts (7). In a survey of 55 isolates (30 from the Nigg strain and 25 from the Weiss strain), Ramsey's group found significant differences in the percent representation of 12 polymorphisms within each strain and between the two strains (8). Interestingly, in vivo passage of a strain results in a significant change in the polymorphism representation compared to in vitro passage, indicating a host selection process. Therefore, the above-mentioned studies suggest that the pathological outcome of an infection is dependent upon both the composition of the infecting chlamydial population (and perhaps even the size of the infecting inoculum) and the genotype of the host, along with
host physiological effects, such as the cyclical production of reproductive hormones.

While individual clonal variants have been phenotypically defined as to their pathological potentials, we know little about how a mixture of variants affects the pathological outcome or the representation of variants within the host. Ramsey’s work clearly suggests that a selection process occurs within the host, but is that selection solely host dependent or is there competition among variants based on variant-specific parameters? We have previously reported that a virulent clonal variant of \textit{C. caviae} strain SP6 and an isogenic attenuated variant, AZ2, individually produce similar courses of infection in guinea pig conjunctiva (9). However, SP6 elicits significantly more inflammation than AZ2. Moreover, when combined in the same inoculum, SP6 outcompetes AZ2, and the pathological outcome is similar to that of SP6 infection alone. In contrast to conjunctival infection, oviduct pathology is dependent upon the ability of the organism to ascend the genital tract. Thus, in the current study, we utilized a similar approach to determine the effect of infection with a mixture of both variants on the ability of each to ascend the genital tract to the uterus and oviduct and to elicit an inflammatory response.

**MATERIALS AND METHODS**

**Experimental animals.** Female Hartley strain guinea pigs, each weighing 450 to 500 g, were obtained from Charles River Laboratories (Boston, MA). All the animals were housed individually in cages covered with fiberglass filter tops, given food and water \textit{ad libitum}, and maintained on a 12-h–12-h light-dark cycle. Each experimental group routinely consisted of 5 to 10 animals. All animal experiments and protocols were approved by the Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

**Genital infection and quantification of infection.** Stocks of the \textit{C. caviae} variants were made according to the standard methodology and were frozen at $-80^\circ\text{C}$ in sucrose-phosphate-glutamate (SPG) buffer until needed (10). The guinea pigs were infected in the genital tract by instilling 50 µl of sucrose-phosphate-glutamate buffer containing $10^6$ inclusion-forming units (IFU) of \textit{C. caviae} SP6 and/or AZ2 directly into the vaginal vault. When both strains were inoculated concomitantly, a suspension was made of equal numbers of IFU of each prior to inoculation into the vagina. SP6 and AZ2 are plaque-purified isolates derived from the conjunctiva of a guinea pig infected with passage 55 of \textit{C. caviae} that have been continually passaged in this laboratory since 1974 (11). AZ2 is resistant to azithromycin (AZM) and was derived by selection in AZM-containing medium (11).

Cervicovaginal swabs for the isolation and quantification of chlamydiae were collected from the genital tract using a Dacron swab that was placed in 2-sucrose-phosphate (2-SP) transport medium with 0.1 mg/ml gentamicin, 0.2 mg/ml vancomycin, and 2.5 µg/ml of amphotericin B (Fungizone) (12). Numbers of IFU were determined by culture in McCoy cells. In order to quantify the AZ2 mutants in mixed infections, swab material was also cultured in the presence of 500 ng/ml AZM (Sigma, St. Louis, MO). The total number of SP6 IFU was then determined by subtracting the IFU obtained in the AZM culture from those obtained in the culture without AZM.

**IL-8 assessment.** To quantify interleukin 8 (IL-8) protein levels in genital secretions, guinea pigs were sedated with ketamine, and absorbent sponges (Ear Wicks; De Royal Industries, Powell, TN), approximately 2 by 10 mm, were inserted into the vagina for 15 to 20 min to absorb secretions. The sponges were frozen at $-70^\circ\text{C}$ until needed. The sponges were then eluted with phosphate-buffered saline (pH 7.4). IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) using a kit for the quantification of human IL-8 (R&D Systems, Minneapolis, MN; Human CXCL8/IL-8 DuoSet, catalog number DY208) that cross-reacts with guinea pig IL-8.

**RESULTS**

**Course of cervical infection.** Initially, we wanted to determine the course of cervical infection in guinea pigs infected with the attenuated AZ2 compared to guinea pigs infected with the virulent SP6. Nine female guinea pigs were infected intravaginally with $10^5$ IFU of SP6, and 10 guinea pigs were infected with AZ2, and the course of the infection was monitored by enumeration of chlamydiae from cervicovaginal swabs. Even though AZ2 has a lower burst size and a lower growth rate than SP6 in vitro, no difference in the course of the infection was seen between the two variants when assessed by two-factor (days and group) analysis of variance (ANOVA) with repeated measures (Fig. 1), similar to our observations in conjunctival infection (9). We had previously observed that AZ2 elicits a weaker inflammatory response than SP6 in the conjunctiva as a result of a decreased proinflammatory cytokine response in the AZ2-infected animals. When we assessed IL-8 levels in genital tract secretions 2 days after genital infection, we also found that IL-8 production in AZ2-infected animals was significantly decreased in comparison to that in animals infected with SP6 ($P < 0.04$; one-tailed $t$ test) (Fig. 2).

**Assessment of ascending infection.** Although there was no difference in the course of lower genital tract infection, we wanted to determine if infection ascending to the oviducts occurred equally with both variants. We had previously observed that viable
**C. caviae** can be isolated in 80% of uterine horns and oviducts by 7 days after infection (15). Therefore, we infected six guinea pigs each with SP6 or AZ2 and removed the uterine horns and oviducts 7 days after infection for isolation of chlamydiae from each tissue. Both uterine horns and both oviducts from each animal were individually assessed for the presence of infection. All tissues were positive for chlamydiae, but interestingly, the number of IFU detected in the uterine horns and oviducts of animals infected with AZ2 was significantly lower than in animals infected with SP6 according to a one-tailed t-test (Fig. 3).

**Infection with a mixed population.** Studies are now showing that chlamydial inocula contain a population of multiple variants of various genotypes with varied pathogenicity (6–8); however, virtually nothing is known about the potential competition of variants within the population or the effect of a mixture of variants upon the outcome of the infection. In order to begin to understand the effect of a mixture of variants on the course of genital tract infection, we infected guinea pigs with equal numbers of the virulent SP6 and the attenuated AZ2 and determined the effect of the mixed population on the representation of each variant in lower and upper genital tract infections. Ten animals each were infected with either 10⁶ IFU of SP6 or AZ2 alone (monoinfection) or 10⁶ IFU each of SP6 and AZ2 (mixed infection). Cervicovaginal swabs were collected on days 3, 6, and 9 postinfection, and five animals from each group were euthanized on day 7 and day 10 postinfection. From each animal, a section of the middle and the distal end of each uterine horn was collected, along with each oviduct, and processed for isolation of chlamydiae. For tissues collected from the group infected with the mixed inoculum, the total number of chlamydiae was assessed, as well as the number of SP6 and AZ2 IFU individually.

As we showed earlier, there was no difference in the course of cervical infection between groups infected with either SP6 or AZ2 alone (Fig. 4). Similarly, the infection course of the SP6-AZ2 mixture group was no different than that of either of the single-infection groups. However, when the numbers of SP6 and AZ2 IFU were quantified in the mixture group, the course of AZ2 infection was lower than that of SP6, although it just missed significance by...
a 2-factor ANOVA with repeated measures (P = 0.06). The number of AZ2 IFU in the mixture group was significantly lower (P = 0.014) than the number of AZ2 IFU in the group infected with AZ2 alone. These data suggest that when combined with a faster-growing variant (SP6), a slower-growing, attenuated variant (AZ2) is at a competitive disadvantage in the mouse, even though in monoinfection it produces an infection comparable to that of the more virulent variant.

When tissues from SP6-infected animals were assessed for the presence of chlamydiae, in contrast to the first experiment, only 70% of the upper uterine horns and oviducts at day 7 were positive while the mid-uterine horn specimen was positive in all of the samples (Fig. 5). Nevertheless, the number of upper tract tissues that were positive in AZ2-infected animals was significantly lower than that in SP6-infected animals according to a Fisher exact test in both the mid-uterine horn set and the oviduct set. Similarly, enumeration of IFU in each tissue showed a significantly greater number of SP6 IFU in both the mid-uterine horn (P = 0.002, according to a Mann-Whitney U test) and the oviduct (P = 0.03) than of AZ2 IFU in animals infected with each individually (Fig. 6). The median number of AZ2 IFU in the upper uterine horns was also lower than that of SP6 IFU (SP6, 4,233 IFU versus AZ2, 0 IFU) but did not reach significance (Table 1). Thus, it remained apparent that AZ2 did not have the ability to ascend the genital tract as readily as the more virulent SP6 in a monoinfection.

The number of IFU from the mixed-infection group on day 7 resembled more closely infection with SP6 alone (Fig. 6). While the number of infected specimens in the mid-uterine horn was significantly less than in the SP6 monoinfection group (P = 0.005; Fisher’s exact test), there were comparable numbers of positive upper uterine horn and oviduct tissues. When total chlamydial IFU in the various tissues of the mixed-infection group were compared to those in each of the monoinfected groups, there was no significant difference in any of the tissues compared to the SP6 group. In contrast, the median total number of IFU in the mixed-infection group was greater in the mid-uterine and upper uterine tissues than in the AZ2 monoinfection group (Table 1). The number of IFU in the oviduct in the SP6 group was significantly greater

![FIG 5](file.png) Percentages of guinea pigs infected with SP6 or AZ2 alone or a mixture of both that were positive for chlamydiae in various genital tract tissues on days 7 and 10 following genital infection. Statistical significance was determined by a Fisher exact test comparing AZ2 and the SP6-AZ2 mixture to SP6 alone in each tissue. Ut, uterus.

![FIG 6](file.png) IFU levels in 10 uterine horns and 10 oviducts in individual animals following vaginal infection with either SP6 or AZ2 alone or a mixture of SP6 and AZ2 on days 7 and 10. Each symbol represents a separate tissue from five animals. Statistical significance was determined by a Mann-Whitney U test.

| TABLE 1 | Median numbers of IFU in different tissue sites |
|---|---|---|---|
| Day | Group | Mid-uterus | Upper uterus | Oviduct |
| 7 | SP6 | 4,233 | 1,482 | 4,000 |
| | AZ2 | 0 | 0 | 0 |
| | SP6 + AZ2 | 5,609 | 3,386 | 9,101 |
| 10 | SP6 | 0 | 0 | 0 |
| | AZ2 | 0 | 0 | 0 |
| | SP6 + AZ2 | 3,810 | 1,905 | 3,704 |
than in the AZ2 group. These data suggested that the presence of a virulent, faster-growing variant may play a more dominant role in the infection outcome resulting from a mixed population.

By day 10, the number of infected tissues in the SP6 monoinfection group had declined from day 7 while the number of infected tissues in the AZ2 monoinfection group increased somewhat, and the mixed-infection group remained comparable to day 7 (Fig. 5). Similarly, the median number of IFU declined in all tissues of the SP6 monoinfection group but remained similar in the AZ2 monoinfection and the mixed-infection groups (Fig. 6) (Table 1). Thus, the differences seen between the SP6 and AZ2 groups were not seen on day 10, suggesting that the infection in the SP6 group had peaked and was on the decline as a result of the immune response. The number of infected tissues and the median IFU levels remained higher in the mixture group than in the AZ2 monoinfection group. The IFU level was significantly higher in the mixed-infection group in the mid-uterine horn tissue. The data suggested that it was likely that the AZ2 and mixed-infection groups were slower in reaching peak levels, perhaps because the host response did not reach a protective threshold as quickly.

We also assessed the numbers of SP6 and AZ2 chlamydiae in the mixed-infection group in the various tissues at days 7 and 10 (Fig. 7). At each tissue site on day 7, the number of SP6 IFU was greater than the number of AZ2 IFU and significantly greater in the cervix and upper uterine horn when compared by a one-tailed paired t test. A similar response was observed on day 10, with significant differences noted in the mid-uterine horn and the oviduct.

We then compared the percentage of AZ2-positive tissues in the mixed-infection group and the percentage of AZ2-positive tissues in the monoinfection group on day 7 to the percentage of positive tissues in the SP6 monoinfection group (Fig. 8). As noted above (Fig. 5), AZ2 in the monoinfection group was present in significantly fewer of the mid-uterine and oviduct tissues than was SP6 (Fisher’s exact test). However, the percentage of AZ2-positive tissues in the mixed-infection group was lower in the mid-uterine horn; there was no difference in the number of AZ2-positive tissues in the upper uterine horn and the oviduct compared to SP6 in the SP6 monoinfection group. On day 10, the number of AZ2-positive tissues was actually slightly higher, although not significantly more than that of SP6 in the SP6 monoinfection group. These data suggest that if a slow-growing, less virulent variant is coupled with a fast-growing, more virulent variant in a population, then the slow-growing variant is more likely to ascend the genital tract than if it is present in a monoinfection.

Flow cytometry analysis of mixed infection. In order to determine the effect of a mixed infection on the inflammatory response, we collected cervical and upper-uterine tissues from the animals in the above-described experiment on days 7 and 10 and assessed the number of PMNs and CD45<sup>+</sup> cells at each site. At 7 days after infection, the numbers of cervical PMNs and CD45<sup>+</sup> cells were lower in AZ2-infected animals and in animals with mixed infection than in guinea pigs infected with SP6 alone, although only the numbers of CD45<sup>+</sup> cells were significantly lower.
and oviduct is dependent upon its virulence. We had previously demonstrated that AZ2 is slower growing with a lower burst size than the “wild-type” SP6 and is unable to elicit a strong inflammatory response in the conjunctiva even though its course of infection is no different than that of SP6 (11). In that study, it appeared that the inability to elicit a strong inflammatory response was associated with a defect in activating the Toll-like receptor 2 (TLR2) inflammatory pathway, resulting in a decrease in expression of tumor necrosis factor alpha (TNF-α) and IL-8 2 days after infection. In the current study, we also observed that the kinetics of the infection course of AZ2 and SP6 in the cervix were identical. In addition, similar to the conjunctival study, IL-8 levels 2 days after infection were lower in secretions of AZ2-infected animals than in those infected with SP6. Moreover, when cervical tissue from AZ2-infected animals was evaluated for PMN and CD45+ cell infiltration, both were decreased compared to SP6-infected animals 7 days after infection. Thus, just as in the conjunctiva, the less virulent AZ2 was unable to elicit an inflammatory response comparable to that to SP6.

Also of interest was the inability of AZ2 to ascend the genital tract to the oviduct as readily as SP6. In one experiment, AZ2 was able to reach the oviducts, but the number of organisms was significantly lower than in infection with SP6. However, in a subsequent experiment, AZ2 was reduced both in numbers of organisms in the uterine horns and oviducts and in the percentage of tissues that were positive for AZ2. The exact reason for the decreased ability of AZ2 to ascend the genital tract remains unknown but may be related to its inability to elicit a strong inflammatory response. We have previously observed by transmission electron microscopy that a major mechanism by which chlamydial organisms are released from host cells is via the detachment of the infected cells by PMNs in both the C. muridarum and C. caviae models (16, 17). When PMNs were depleted in mice, the number of chlamydial organisms increased significantly in the cervix (16). In addition, Imtiaz and colleagues noted that in mice deficient in matrix metalloproteinase 9 either genetically or by antibody-mediated depletion, chlamydial organisms are unable to ascend the genital tract, even though chlamydial numbers in the cervix remain unaltered (18). The data from these studies strongly suggest that an inflammatory response is essential for ascending infection to occur and that in vivo, PMNs are an integral component of the chlamydial strategy for spread to new host cells and distribution to new sites. The inability of AZ2 to elicit a robust inflammatory response that is associated with decreased activation of the TLR2 pathway could explain its decreased ability to ascend the genital tract (9). The lower growth rate of AZ2 could also be a factor in reduced ascending infection; however, the kinetics of AZ2 infection versus SP6 infection in the cervix do not support this explanation. Lei and colleagues noted decreased survival of plasmidless C. muridarum in the upper genital tract (19), and that could have been a possibility in our study as well, since AZ2 is not as “fit” as SP6 in vitro (11). While the competition studies discussed below support the fitness explanation, reduced ascending infection may be due to a combination of decreased inflammation and decreased fitness of AZ2.

The findings that were of the greatest interest in our study were the effects of a mixed infection with equal numbers of virulent and attenuated organisms. Virtually all studies in the genital tract models of chlamydial infection have utilized either an undefined stock population of chlamydial organisms or a plaque-purified clonal organism. There have been no studies of genital tract infections with
defined populations with respect to variant composition. We are well aware that the constructed population that we used in our study was highly artificial, using equal amounts of only two variants in contrast to the natural situation in which multiple variants are likely present in varying ratios. Nevertheless, the outcome of our experiments marks a starting point for understanding how chlamydial variants compete within the context of an ascending genital tract infection. Similar to what we observed in the conjunctival model (9), the total number of chlamydiae in the cervix in the first 9 days postinfection in a mixed infection with SP6 and AZ2 was comparable to those in a monoinfection of each variant alone. However, of interest was our observation that SP6 outcompeted AZ2 in the mixture and by 9 days postinfection represented 92% of the total number of chlamydiae in the cervix. The number of AZ2 IFU in the mixed infection was significantly lower than that of AZ2 at 9 days in a monoinfection; thus, it is unlikely that the growth rate of AZ2 alone can account for their decreased number in the mixed infection. We also observed in the conjunctival model that within the mixed-infection group, SP6 was always significantly more numerous than AZ2, although there was no difference in monoinfections of each (9). It would appear that the presence of SP6 in the mixture was a key factor in the reduced number of AZ2 IFU. One explanation is that the two variants are competing for a limited number of host cells, and SP6, being faster growing and producing a greater yield of elementary bodies, would have an advantage. In a recent publication, we demonstrated experimentally and mathematically that the number of available host cells can dramatically influence the course of infection (20). Even if a host cell were infected with both variants, SP6 would have the competitive advantage. As we have previously suggested (9), because of its advantageous growth characteristics, SP6 may be able to produce enough progeny to compensate for organisms lost to killing by PMNs. On the other hand, AZ2, because of its reduced growth rate, may suffer a higher proportion of killing by PMNs, further shifting the balance to SP6.

Because of the competitive advantage of SP6, it was surprising that in the mixed infection, we observed increased numbers of AZ2 in the upper genital tract, rather than SP6 alone. Overall, the number of chlamydiae with the mixed infection in the uterine horns and oviducts did not differ from that in guinea pigs monoinfected with SP6 but was much greater than in animals with an AZ2 monoinfection. Nevertheless, the percentage of animals with AZ2, as well as the number of AZ2 IFU in the upper genital tract in the mixed infection, was markedly greater than in the AZ2 monoinfection, suggesting that the presence of SP6 facilitated AZ2 ascension of the genital tract. If our hypothesis that the inflammatory response is critical for chlamydiae to ascend the genital tract (16–18) is correct, then the inflammatory response induced by SP6 would not only be selective for a given variant (SP6 in this case) but would also facilitate ascension up the upper genital tract of all variants in the population, including the less virulent ones. Thus, the data in this study provide additional evidence to support the role of PMNs in effecting the spread of chlamydial infection both proximally and distally in the female genital tract.

What, therefore, is the significance of the competition among variants? Perhaps, most importantly, it is possible that transmission from individual to individual selects for more virulent variants. Traditionally, if one wanted to increase the virulence of a bacterium, one would do sequential passages through animal hosts, theoretically to select for those variants more adapted to living in a host. Recently, Ramsey and his group have demonstrated that the sequential genital tract passage of both the Weiss and Nigg strains of C. muridarum in mice resulted in a significantly altered quantitative representation of variants compared to the initial tissue culture passage preparation (8). In contrast, but supporting this concept, Zhong’s group recently reported that the in vitro passage of C. muridarum also shifted the variant representation but, in their study, toward a less virulent phenotype (21). Then, in “real life,” it seems that since transmission among individuals is not interrupted by a tissue culture passage, one would expect that all individuals would acquire the most virulent or host-adapted set of variants. However, it is clear that not all individuals have the same disease outcome, with the genetic makeup of the host potentially having a major impact on selection. Both immune and physiologic parameters are different in each individual based on host genetics, so that a given variant(s) may be preferentially selected because of specific bacterial physiologic parameters and requirements, including but not restricted to replicative ability and ability to elicit an inflammatory response. Our study placed two contrasting variants together, while in nature, there would be multiple variants, many of which may not differ as drastically. Nevertheless, the differences between a mixed infection of two variants and the two in monoinfections are striking and indicate that, as in any population in nature, infection in an animal is accompanied by selection and an ebb and flow of specific phenotypes and genotypes of the infecting microbe.

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