Effect of Chlamydiaphage φCPG1 on the Course of Conjunctival Infection with “Chlamydia caviae” in Guinea Pigs

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Over the last several years, four different phages of chlamydiae, in addition to a phage associated with Chlamydia psittaci isolated from an ornithosis infection in ducks over 25 years ago, have been described and characterized. While these phages and their chlamydial host specificities have been characterized in vitro, virtually nothing is known about the interaction of the phage with chlamydiae in their natural animal host. φCPG1 is a lytic phage specific for “Chlamydia caviae,” which is a natural parasite of the guinea pig. In this study, guinea pigs were inoculated in the conjunctiva with suspensions of φCPG1 and C. caviae and the effect on the development of pathology and on the course of chlamydial infection in the conjunctiva was determined. The presence of phage delayed the appearance of the peak level of chlamydiae in the animal and decreased the pathological response. Evidence for replication of the phage in C. caviae in the conjunctival tissue was observed. Modifying the ratio of phage to chlamydiae altered the course of infection and affected phage replication. There was no evidence for the phage increasing the virulence of C. caviae infection.

Over the last several years, four different phages of chlamydiae, in addition to a phage associated with Chlamydia psittaci isolated from an ornithosis infection in ducks over 25 years ago by Richmond and coworkers (22), have been described and characterized. Hsia et al. isolated phage R-22 (22) and φCPG1 from in vitro-cultured “Chlamydia caviae,” the agent of guinea pig inclusion conjunctivitis (7, 8). This was followed shortly thereafter by the isolation of phages Chp2 (2) and φCP01 (R.-C. Hsia and P. M. Bavoil, unpublished data) from Chlamydia abortus, φAR39/φCpn1 from Chlamydia pneumoniae (10, 18), and Chp3 from Chlamydia pecorum (3). The chlamydiaphages belong to a class of lytic phages that include the closely related Bdellovibrio bacteriovorus phage pMH2K (1) and the Spiroplasma meliferum phage SpV4 (21) and for which the single-stranded DNA coliphage φX174 is the prototype (4). The chlamydiaphage host range for the various chlamydiaphages is varied, but all are lytic for their respective hosts; however, it is interesting to note that chlamydiaphages have not yet been detected in association with Chlamydia trachomatis or Chlamydia muridarum.

That five different chlamydiaphages have been described for chlamydiae infecting different animal species from geographically distinct areas would suggest that they are prevalent in chlamydiae in nature. Nevertheless, because all of the chlamydiaphages have been isolated from chlamydiae in tissue culture and their interaction with their hosts has been characterized from tissue culture, there is literally nothing known about the interaction of the chlamydiaphages with their bacterial hosts in the context of infection of the natural hosts of the bacteria. In fact, there are no data describing the interaction of any bacteriophage of an obligate intracellular bacterium in the natural animal host of the bacterium.

It is indeed fortunate that φCPG1 has been isolated from C. caviae since C. caviae is a natural parasite of the guinea pig and a model for both chlamydial ocular and genital tract diseases. This presents the opportunity to evaluate the role of chlamydiaphage in the pathogenesis of chlamydial infection in vivo. While phage-bearing strains of bacteria have often been found to be more virulent than the same strains without phage because of the presence of virulence genes in the phage, there is no evidence to date of any genes associated with the φCPG1 genome that impact chlamydial pathogenicity. Moreover, since the current strains of C. caviae were isolated from guinea pigs over 40 years ago (12), there is no published evidence for the detection of φCPG1 in vivo in the animal host. Thus, in this study, we undertook to determine the effect of chlamydiaphage infection of C. caviae on the course of chlamydial conjunctival infection in the guinea pig model and to determine the viability and infection kinetics of the chlamydiaphage in the conjunctival tissue.

MATERIALS AND METHODS

Propagation of chlamydiae. C. caviae cultured in the Rank laboratory at the University of Arkansas for Medical Sciences (UAMS) was originally obtained from the late Edward Murray as a direct passage of the original isolate from guinea pigs (12) and has been continuously passaged since that time. This stock has been found to be phage free when evaluated by PCR for the gene encoding the major capsid protein, VP1. In fact, it should be noted that the original passage (passage 1) in Murray's laboratory from guinea pigs in 1962 (12), obtained from the late Bruce MacDonald, was also found to lack the phage. C. caviae for infection purposes was grown in McCoy cells, and stocks were prepared according to previously described techniques (5). Only phage-free C. caviae was grown in the UAMS laboratory.

Propagation of chlamydiaphage. All chlamydial stocks containing phage were grown in the Bavoil laboratory at the University of Maryland and sent to UAMS...

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for direct inoculation into guinea pigs without further manipulation. ϕCPG1 phage was originally isolated from C. caviae cultured from guinea pigs by Julius Schachter (6). This C. caviae isolate was a distinct isolate from guinea pigs and was obtained from Schachter in 1987 and maintained in the Bavoil laboratory. When it became apparent that the Schachter C. caviae was infected with ϕCPG1, the Murray strain of C. caviae was given to P. M. Bavoil in 1993 by R. G. Rank. All of the subsequent phage culture has been performed with the Murray C. caviae isolate. Thus, the Murray C. caviae strain has been maintained separately in the Rank and Bavoil laboratories since that time.

C. caviae/phage culture lysates were prepared mostly as previously described (6). Briefly, 10 plates of confluent HeLa cells were infected with 6.4 × 10^7 inclusion-forming units (IFU) of phage-free C. caviae preinfected with a CHCl3-treated C. caviae phage lysate in sucrose-phosphate-glutamic acid (SPG) buffer. After incubation for 2 h with intermittent rocking at room temperature, Dul-becco’s modified Eagle medium was added, and incubation continued for 11 days at 37°C in 5% CO2. The resulting C. caviae/phage culture lysate is highly enriched for phage but also contains 10^6 IFU/ml of C. caviae because not all of the chlamydiae become infected. Purified phage was obtained upon treatment of C. caviae/phage culture lysates with chloroform (1:1). The chloroform suspension was immediately centrifuged at 10,000 × g at 4°C for 5 min, and the aqueous layer was transferred into a clean tube. This suspension was again centrifuged in order to remove the remaining chloroform.

Inoculation of guinea pigs. Female Hartley strain guinea pigs (Charles River Laboratories), weighing 500 to 550 g, were inoculated ocularly by depositing 25 g/ml of C. caviae because not all of the chlamydiae become infected. Purified phage was obtained upon treatment of C. caviae/phage culture lysates with chloroform (1:1). The chloroform suspension was immediately centrifuged at 10,000 × g at 4°C for 5 min, and the aqueous layer was transferred into a clean tube. This suspension was again centrifuged in order to remove the remaining chloroform.

Quantitative PCR for phage detection. Because ϕCPG1 is a phage of an obligate intracellular bacterium, standard methodology for the quantification of phages of other bacteria could not be applied here. A separate swab was collected and placed in 1 ml of SPG buffer to determine the amount of phage genomic DNA. Ten microliters of this suspension was processed directly for preparing DNA with Sigma Extract-N-Amp blood PCR kits. The processed sample (2.5 μl) was used for quantitative PCR in a reaction mixture containing 0.4 μM of each primer and probe in a 12.5-μl reaction volume containing iQ supermix (Bio-Rad). The primers used for amplifying ϕCPG1 are Hex-AGGCT CTTCAACATCAACATTCA (Forward) and TCTTGTTGACCACTTATATCA (Reverse). None of the Fluor dyes or quencher was used. Quantitative PCR was carried out using iQ supermix in a Bio-Rad iCycler, following the protocol of 95°C denaturation for 3 min, followed by 40 cycles of 95°C (30 s) and 60°C annealing (1 min). A standard curve was generated with recombinant phage DNA (plasmid pCpG1) (7). Absolute concentrations of DNA obtained from PCR were extrapolated to whole swabs for representation in figures. These primers do not amplify the residual phage DNA which is incorporated in the C. caviae genome.

Assessment of pathological response. Gross pathological changes in the conjunctiva were evaluated daily following inoculation. Both eyes from each animal were observed, and the score was recorded; thus, the pathology score represented the mean of 10 conjunctiva in each group. There was generally very good agreement in the scores from both eyes in individual animals. The changes were assessed on a scale of 0 to 4+ as previously described (16). Briefly, palpebral and bulbar conjunctivas were evaluated for erythema, edema, and exudation. The scores were defined as follows: slight erythema or edema of either the palpebral or bulbar conjunctiva, 1+; definite erythema or edema of either the palpebral or bulbar conjunctiva, 2+; definite erythema or edema of both the palpebral and bulbar conjunctivas, 3+; definite erythema or edema of both the palpebral and bulbar conjunctivas and the presence of exudate, 4+.

Antibody response. Terminal bleeds were obtained from each animal, and the presence of antibody to the VP1 protein of ϕCPG1 was determined by immuno blot analysis. VP1 (the major capsid protein) is the most abundant phage protein, is a potent immunogen, and is considered immunodominant in the context of infection. Briefly, Escherichia coli BL21 cells carrying pET30a/VP1 or pET30a were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biosciences). Immune sera from ϕCPG1-infected C. caviae-infected guinea pigs were preadsorbed with lysates of E. coli BL21(pET30a) to remove nonspecific antibody (not shown). Membranes were blotted with the preadsorbed sera diluted 1:100, followed by alkaline phosphatase-conjugated donkey anti-guinea pig immunoglobulin G polyclonal antibody (Chemicon). The blots were visualized with ECF (Amersham Pharmacia Biosciences) and read using ImageQuant 5.2 (Amersham Biosciences).

Statistical analyses. Since all experiments recorded repeated measures from individual animals, statistical significance was determined by a two-factor (group, days) analysis of variance with repeated measures on one factor (days). Differences were considered to be significant if P was <0.05.

RESULTS

Because there is no information available on the relative numbers of phage particles associated with chlamydial infection in vivo, we arbitrarily decided to first inoculate animals with the C. caviae/phage culture lysate (Bavoil laboratory). The lysate contained approximately 10^9 IFU/ml of chlamydiae and 51.44 μg/ml of VP1 DNA, as determined by quantitative PCR. As controls, groups were inoculated with 10^6 IFU of C. caviae or chlamydialphage alone. Each experimental group contained five guinea pigs. The chlamydialphage alone was derived from an aliquot of the C. caviae/phage culture lysate. A large amount of phage DNA was lost in the purification process so that the chlamydialphage inoculate contained 4.14 μg/ml of VP1 DNA.

The gross pathological response was assessed at various times after inoculation. The conjunctivas inoculated with phage-free C. caviae began to develop pathology by 2 days after inoculation and reached a peak response by 4 days, but pathology subsided thereafter and finally resolved by 12 days after inoculation (Fig. 1A). In contrast, the ocular response in animals inoculated with the C. caviae/phage culture lysate was significantly different from the control (P < 0.001 according to a two-factor [group, days] analysis of variance with repeated measures). There was a significant delay in the onset of the pathological response in animals inoculated with the C. caviae/phage culture lysate, with conjunctivitis first being detected 5 days after inoculation. The response peaked at 6 days but did not achieve the same level of intensity as that for the control phage-free C. caviae preparation. No gross pathology at any time was observed in animals inoculated with chlamydialphage alone, indicating that any residual C. caviae was not infectious or that the dose was below the 50% infectious dose (48 IFU) for C. caviae in the eye.

The courses of C. caviae infection in both groups of animals were evaluated by the culture of conjunctival swabs collected every 3 days. Because the C. caviae inoculum was relatively high, the level of IFU in the phage-free C. caviae group reached high levels very quickly and then gradually diminished until day 21 (Fig. 1B). The infection in guinea pigs inoculated with the C. caviae/phage culture lysate was significantly different from that in the control group (P < 0.001), increasing more slowly and peaking 3 days later than that in the control group, although both groups resolved their infections similarly. Thus, it was apparent that the presence of the phage modified the course of chlamydial ocular infection and also reduced the pathological response to C. caviae.

In order to determine whether ϕCPG1 was able to replicate in vivo, the amount of phage DNA was quantified from the
conjunctival swabs by quantitative PCR. Comparable to chlamydial IFU quantification, phage DNA was detected 3 days after inoculation and then decreased thereafter and was not detected after day 9 (Fig. 1B). The early detection of phage DNA suggested that many of the C. caviae cells probably became infected very quickly after inoculation into the conjunctiva or had already been infected in culture. This experiment was repeated with similar results (data not shown).

We next wanted to determine the effect on the infection course if phage was added to a stock of C. caviae just prior to inoculation into the animal. There was also some concern that the C. caviae cells maintained in the Bavoil laboratory that were used and contained in the C. caviae/phage culture lysate may differ somewhat in pathogenicity from the C. caviae cells maintained in the Rank laboratory. Therefore, phage from a stock containing 10^6 IFU of C. caviae was treated with chloroform to kill the bacteria and then, after a washing, was combined with a fresh stock of 10^6 IFU of C. caviae (Rank laboratory) (C. caviae plus phage). Hence, the C. caviae contained in the mixture with the phage was from the same source as the C. caviae inoculated into control animals. This preparation was inoculated into the conjunctiva as before. Just as in the animals inoculated with the C. caviae/phage culture lysate, there was a significant delay (P < 0.001) in the time to the development of the peak pathological response in the conjunctiva (Fig. 2A). Moreover, the degree of response did not attain the level of the pathology in animals infected with phage-free C. caviae. Again, there was no pathological response to inoculation with phage alone. There was also a significant difference (P < 0.001) in the courses of the infection, as measured by isolation of chlamydiae from the conjunctiva, with chlamydiae in the phage-inoculated group increasing more slowly (Fig. 2B). In this experiment, there was clear demonstration of replication of the chlamydiaphage in vivo (Fig. 2B). Phage DNA was present at a low level at 3 days after infection but increased to a peak level at 6 days and then rapidly declined. The peak phage activity occurred concomitantly with the peak IFU count, suggesting that, as the C. caviae population expanded, so did the phage population. This experiment was repeated as part of the next experiment (see Fig. 3, C. caviae plus phage, 1:1 group).

Because we arbitrarily chose the relative amounts of C. caviae and phage in the original stock preparation as the inoculum in the first two experiments, we wanted to determine if the courses of both the chlamydial and phage infections would be different if we modified the ratio of chlamydiae to phage in the infection inoculum. Therefore, we inoculated three groups of five guinea pigs each in the conjunctiva with either a 10:1 ratio of C. caviae/IFU to phage (10^6 IFU C. caviae and a 1:10 dilution of the phage preparation), a 1:10 ratio of C. caviae IFU to phage (10^5 IFU C. caviae and undiluted phage), and the same ratio as in the previous experiments (10^6 IFU C. caviae and undiluted phage). Two additional groups of animals were inoculated with either 10^5 or 10^6 IFU of phage-free C. caviae. The course of the infection was monitored by conjunctival swabs at 3-day intervals.

The group infected with C. caviae/phage at a 1:1 ratio had a significantly reduced level of C. caviae (P < 0.015) compared to the group infected with 10^6 IFU of phage-free C. caviae, similar to the previous experiments (Fig. 3A). The level of
likely that the clearance of chlamydiae reduces the C. caviae pool available for phage infection. However, we wanted to determine whether there was also an immune response to the phage alone. Terminal sera from the animals, approximately 35 days after infection were evaluated by immunoblot analysis against the VP1 phage protein to determine if antibody was elicited. There was only weak antibody to phage detected in a few of the sera collected from the animals infected in the conjunctiva with C. caviae and phage (data not shown). While VP1 is the most abundant phage protein and clearly immunogenic (see below), there is really a paucity of information about the immune response to phage infections in their natural infectious-disease setting. Therefore the possibility that the phage harbors other strong antigens cannot be excluded. However, sera collected from two animals inoculated intranasally with the C. caviae/phage preparation at the same levels as the stock preparation were strongly positive for antibody to VP1, demonstrating that the phage was indeed immunogenic. The apparent lack of an antibody response in the conjunctiva may be related to inaccessibility of sufficient phage antigen to the host immune system to be immunogenic.

FIG. 3. Effect of different ratios of chlamydiae and phage on chlamydial infection in the conjunctiva. (A) C. caviae (10⁵ IFU) and purified phage from the original C. caviae/phage culture lysate or a 1:10 dilution of the purified phage from the lysate were inoculated into the conjunctiva. (B) C. caviae (10⁵ IFU) and purified phage from the original phage stock were inoculated into the conjunctiva. Each group contains five animals each, and the data points represent the means for each group. The error bars for the phage DNA represent 1 standard deviation.

This study marks the first report characterizing the effect of a phage infection of its obligate intracellular bacterial host in a local-tissue site in the natural mammalian host of the bacterium. Previous in vitro studies have documented the impact of phage infection on C. caviae (6, 7). A notable feature of phage-infected C. caviae is its developmental arrest at the reticulate body stage accompanied by the appearance of aberrantly enlarged reticulate bodies similar to forms induced upon a variety of stresses (e.g., nutrient or iron deprivation or gamma interferon or penicillin treatment). Phage-induced lytic death of these chlamydiae resulted in immediate lysis of the inclusion and of the host cell. This dramatic impact of phage infection on both its prokaryotic and eukaryotic hosts suggested that phage infection might also provoke an altered pathological outcome. It appeared that the primary effect of qCPG1 infection on the course of chlamydial infection was in delaying the onset of the peak level of infection. While the numbers of IFU ultimately attained by phage-infected cells and controls were the same, the number of IFU in the early time points was about 2 logs lower than those at the comparable time point in animals without phage. A consequence of the early reduction of chlamydiae in the conjunctiva was a reduced pathological response, regardless of the inoculum dose, the peak pathological response occurs about 5 to 7 days after infection. Decreasing infection doses merely lower the peak intensity of the pathological response (R. G. Rank, unpublished data). This suggests that the number of chlamydiae present early in the infection is critical in eliciting the inflammatory response. Thus, phage killing of...
chlamydiae early in the infection could explain the decreased pathological response. One might speculate that killing of chlamydiae by phage may release more chlamydial and cellular debris and perhaps exacerbate the inflammatory response, but this does not seem to be the case.

An alternative but equally viable explanation is that, since phage infection of chlamydiae dramatically alters the physiology of the chlamydiae by inhibiting replication and otherwise disrupting the bacterium and host cell (6), there could be significant interference with signaling of the host cell by the organism, thereby reducing the chemokine and cytokine response, resulting in a decreased inflammatory response. An early reduction in the inflammatory response has the added cascading effect of decreasing the chemokines and cytokines produced by PMNs because there would be fewer PMNs at the site. We have observed in vivo in the mouse model of chlamydial infection that the chemokine and cytokine response responsible for acute inflammation at 12 to 24 h after infection requires viable chlamydiae and is not induced by killed organisms (15). Thus, interference of the phage with normal physiologic pathways and organism interactions with the host cell could have significant effects on the development of the inflammatory response. Nevertheless, the exact mechanism of the reduced pathological response remains to be determined.

While there are many examples of increased pathogenicity of bacteria through the introduction of virulence genes by lysogenic phage, this does not appear to be the case with *C. caviae*. Remnants of the phage genome can be detected in the *C. caviae* genome, but these are incomplete and nonfunctional (20). Interestingly, PCR analysis of a DNA sample from the first passage of the Murray isolate in 1962, which has been archived in the Rank laboratory, reveals the presence of a conserved phage sequence in the *C. caviae* genome (data not shown), suggesting that phage was indeed associated with *C. caviae* in natural infection in guinea pigs. The presence of the conserved phage sequence in the genome may be the stable result of multiple ancient recombination events, which at some stage in *C. caviae*’s evolution may have served a now long-lost functional purpose. It is interesting to note that a different segment of the closely related phage φAR39 genome also exists in the *C. pneumoniae* genome (11). Thus far, there has been no evidence for new integration of phage DNA into the chlamydial genome in vitro studies. Based on our observations of reduced pathology in vivo, there does not appear to be any overt effect of enhancing chlamydial virulence; to the contrary, the phage appears to reduce the pathological response by killing chlamydiae.

An interesting aspect of this study is that the phage infection is not present for a very long period of time and appears to resolve more quickly than does the chlamydial infection. Modifying the dose of phage with respect to *C. caviae* does not alter the level and kinetics of the phage and chlamydial infections to any great extent. The resolution of the phage infection is likely dependent upon the decreasing numbers of chlamydiae, and in particular the accessibility of the now-rare elementary bodies to infectious phage particles. In vitro, it has been observed that phage gains access to susceptible reticulate bodies within the intracellular inclusion by first attaching to the extracellular elementary body (6). In an ongoing chlamydial infection of the conjunctiva, there is a substantial acute inflammatory response, resulting in tissue destruction. Moreover, we have recently observed by transmission electron microscopy that PMNs appear to facilitate the shedding of infected host cells from the conjunctival epithelium (17) and that many of these detached cells still have immature and intact chlamydial inclusions within the cells. We also observed that any elementary bodies which were not in inclusions had already been phagocytized by PMNs. Thus, in the milieu of an ongoing inflammatory response, it may be difficult for phage to “find” and attach to free elementary bodies. In addition, the intense inflammatory response may be detrimental to the phage itself. Thus, it is not surprising that the phage infection of *C. caviae* in vivo is short-lived.

With this in mind, how then has chlamydia-phage persisted in nature if its survival is solely dependent upon its association with *C. caviae*? If the target tissue of *C. caviae* is the conjunctiva, it is very clear that the infection resolves in about 3 weeks and then animals have a high degree of immunity to reinfec- tion for relatively long periods of time, making a scenario of continuous animal-to-animal transmission strictly via mechanical transmission of conjunctival secretions unlikely over evolu- tionary time. Although it has not been confirmed, it is likely that *C. caviae* infection, as in most of the other nonprimate chlamydial infections, is a natural infection of the guinea pig gut. Murray isolated *C. caviae* from the eyes of young guinea pigs but not from mature animals (12), so it is conceivable that *C. caviae* also persists in the guts of guinea pigs and only infects the eye in newborn animals exposed to fecal contaminants. It is possible that chlamydiae may persist in the gut in the absence of an inflammatory response. In fact, Igietseme and coworkers were able to isolate *C. muridarum* from rectal swabs of mice for up to 8 months after oral infection but could not demonstrate a pathological response in the gut (9). Such a situation would be amenable to the persistence of the chlamydia-phage relationship in vivo.

Another nonexclusive possible explanation for phage survival in the face of potent immune responses against itself and its host chlamydiae is that the phage is readily able to alter its host specificity through mutation of its coat protein(s). We previously identified IN5, a surface-exposed 71- to 85-amino-acid-residue loop of the VP1 major capsid protein, which appears to mediate host specificity of the chlamydiaphages (19). The sequence of this segment in φCPG1 differs significantly from that in phages SpV4 and φMH2K, which, respectively, infect *Spiroplasma* and Bdellovibrio but is relatively conserved with respect to that in Chp2 and nearly identical to that in φAR39 (one K-to-Q change), which infects *C. pneumoniae*. The genomic sequence of φCPG1 is in fact virtually identical to that of the *C. pneumoniae* phage φAR39. Moreover, φCPG1 was able to infect *C. pneumoniae* (the phage-free strain CWL029) while conversely φAR39 was able to infect *C. caviae* in vitro experiments (Hsia and Bavoil, unpublished).

While the guinea pig conjunctiva may not be the natural site of phage persistence in association with chlamydiae, the present study does demonstrate that φCPG1 can indeed infect and replicate in *C. caviae* in the natural animal host for *C. caviae*. It does not have any apparent enhancing effect on chlamydial virulence and may actually be a factor in controlling a chlamydial population and downregulating the pathological response at its natural tissue target.
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