Influence of exogenous agents on the expression of *Neisseria gonorrhoeae* variants that survive the death of the parent culture.

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Abstract

When one grows *Neisseria gonorrhoeae* as a mat on agarose media, small foci of viable cells develop within the mats that are darker in color and survive after the surrounding parent cells have died. The addition of bovine serum albumin, RNAse, lysozyme, and a non-gonococcal origin DNA suppressed the frequency of these foci in the mats. Only one of the agents that was tested, DNAse I, increased the frequency of foci. The effects of these biological agents on modulating bacterial variation is consistent with them acting on the bacterium's uptake of autogenous DNA. All of the tested agents have analogs in the human host, suggesting they have some role in innate immunity. That extracellular agents were able to suppress the expression of new variants suggests a novel approach to developing vaccines and anti-bacterial compounds to control gonococcal infections.

Neisseria gonorrhoeae and other pathogenic bacteria have the capacity to change phenotype, a property sometimes referred to as phase variation (12). In their early work, Kellogg et al. observed that when fresh cultures of *N. gonorrhoeae* were grown *in vitro*, they exhibited colony morphologies that corresponded to their degree of piliation (6). Later, others noted phase variation in several of its other outer membrane proteins such as the opa protein (11). There seems to be a consensus that phase variation is a mechanism by which the bacterium eludes the immune system and it does this by presenting an ever-changing antigenic profile (1, 12). Yet, while researchers have worked out many of the molecular mechanisms by which the bacterium accomplishes these phase shifts (12), we know little about the "survival" variants described here or the effect of the milieu in which these and other variants arise.

Another important aspect of infections is that bacteria form biofilms as part of the pathogenic process. Only recently have people begun to study phase variation in biofilms (3). The first studies, while informative, have yet to address all the dynamics of variant expression in biofilms, particularly during the initial colonization events and they offer little information about how to terminate the unwanted colonizations we call infections. The present study grew out of my observation that when *N. gonorrhoeae* grows as a mat on agar media, foci of viable bacteria appeared after a few days within the moribund parental culture. This led me to explore the effect of adding agents, such as homologs of proteins found in the human host's body fluids, into the solid growth medium to see if they might affect the emergence of new phase variants in these temporary biofilms.

Materials and Methods

Because most of the agents tested were proteins and subject to denaturation when heated, I performed all the experiments using an agarose-based medium that could be cooled sufficiently before the proteins were added, mixed, and allowed to set into a gel. The basic agarose media contained 1.5 g proteose peptone #3 (Difco, Detroit, MI), 0.4 g K2HPO4, 0.1 g KH2PO4, 0.5 g NaCl, 0.5 g low EEO agarose (Boehringer-Mannheim), and 100 ml water. This medium was autoclaved for 20 min before adding 1 ml of Kellogg's supplement (6) after the medium had cooled to 40°C. The proteins that were added were bovine pancreatic DNAse I (01. mg/ml; Sigma), bovine pancreatic RNAse (0.1 mg/ml; Sigma), hen egg white lysozyme (50 μg/ml; Sigma), and bovine serum albumin (50 μg/ml; Sigma). These were filter sterilized through a 0.45 μm filter prior to addition to the agarose medium in a volume of 1 ml per 100 ml of medium to limit dilution of the medium. Control media were prepared by the addition of filtered water or carrier solution in place of the filtered agent. Because DNA is less sensitive to heat, one could add the salmon egg DNA (2 μg/ml; Difco) to the medium as a dry powder before the autoclave step. A measured amount of 20 ml of the medium was poured into each 100 mm diameter plastic Petri dishes to ensure even thickness. The media were cured for at least 48 hr before use.

To create the gonococcal mats, 40 μl of a suspension of the non-piliated form of *N. gonorrhoeae* was dropped onto the agarose surface. When you do this, the drop spreads into a circle of about 2 to 2.5 cm diameter. One can grow four mats in one Petri dish. The innoculum was prepared by swabbing the growth off standard GC medium base agar into GC medium base broth to a turbidity (A_{600}) of 1.7 which is approximately 4 ×

10^9 cfu/ml. The mats were incubated at 35°C with 5% CO_2. In all cases, there was more than a 48 hr lag before any variants began to appear in the mats. The variants appeared within the mats as foci of small diameter (Fig. 1). When incubated longer than 72 hours, the only viable bacteria that could be isolated were from the mat's edge and the variant foci. The viability of the bacteria in the foci and in the surrounding parent culture was tested both by direct sampling of these sites with an inoculating needle as well as by replica plating. The number of foci in each mat was counted twice a day for 4 to 5 days. If there were large numbers of foci, they were counted with the aid of a stereoscopic microscope at low magnification. Because the bacteria at the very edge of the mats remained viable, the foci that developed within approximately 2 mm of the edge were not counted.

Results

While every isolate gave rise to these variants, the average number depended on the choice of bacterial isolate and its passage history. For some isolates, the foci appearing in the mats without any reagent addition were so numerous as to make counting them impractical, while in others so few appeared that it would have required the counting of the foci in a very large number of mats to achieve statistical significance. The variation in foci number from isolate to isolate seemed to correlate with the history of the isolate. In a direct comparison of mats formed by three isolates, relatively few foci (7.3 ± 1.2) developed within the mats (N = 8) formed by the Pgh3-1 isolates at 72 hours; while the number that appeared within the mats (N = 7) formed by the I-756 isolate were too numerous to count. The number forming within the LB-2 isolate mats (N = 8) was

intermediate (43.3 ± 11.3). Unfortunately, we do not know the exact number of *in vitro* passages for any of these isolates. However, there seems to be a correlation with usage history in the laboratory. The Pgh3-1 has been the object of study stretching back to the 1970's and has been subject to a large number of passages over the years (2), while the I-756 isolate which was isolated at about the same time has little history of use in research. The LB2 isolate, which has a history of moderate passage, proved the most practical for this study since a sufficient number of foci appeared in its mats to allow a good statistical examination of the data from a relatively small number of mats. Still, even the LB2 isolate exhibited variation in foci frequency within its mats from experiment to experiment. The number of foci developing within the mats declined with the number of passages from the frozen culture. In an experiment comparing the number of foci developing in mats (N = 8) of the LB-2 isolate passaged only once, 230 ± 21.3 foci appeared, but after three passages, this dropped to 49 ± 3.4 foci per mat. Because the number of foci developing in the LB-2 mats tended to be intermediate in number, this isolate was chosen for the experiments with the exogenous agents.

In all experiments, there was a lag time of at least two days before the appearance of any foci (Figures 2 through 3). This may be partly due to parental culture repression (8). Parental repression occurs when the parent bacterial colony is proliferating at such a rapid rate, any new variant within it has difficulty achieving expression. I suspect this is due to the lag time needed by the nascent transformants to enter into their normal replicative rate. Whatever the cause, the new variants in the *N. gonorrhoeae* mats appear when the rate of growth of the parent culture begins to wane and starts to die. However,

since the bacteria in the foci outlive the parent culture these new variant bacteria may be an adaptation to stress.

To explore the effect of the various exogenous agents on the appearance of new variants, I added them to the medium and counted the number of foci appearing in the mats. These were then compared to the number that developed in mats of the bacteria from the same inoculum grown on the same batch of medium to which a filtered carrier solution was added. Both hen egg white lysozyme and bovine serum albumin repressed foci expression (Figure 2). The repression of foci due to each agent occurred in a dose dependent manner (Data not shown). A special comment must be made about lysozyme, which has intrinsic bactericidal activity for some bacterial species. It did not exert detectable bactericidal activity during the first 48 hours of mat growth and its influence on foci expression was not statistically different from that of BSA at the same weight per volume concentration. The addition of RNAse to the medium had a comparable effect as that of albumin and lysozyme. When I added DNAse I to the media, however, there was quite a different result than for any of the other proteins (Figure 3). Although DNAse I represses transformation in some strains (4, 14), this was not seen for any of the isolates in this study. Instead, it greatly increased the expression of foci within the mats. In contrast, the presence of the non-bacterial origin DNA from salmon egg in the medium repressed foci expression (Figure 4) confirming earlier observations that autogenous origin DNA is preferred for the generation of new variants (10).

Discussion

It was not surprising that new variants arise from the uptake of autogenous DNA derived from sibling bacteria that have either secreted DNA into the medium (9) or have lysed. However, the bacteria seem to import DNA more efficiently when a DNAse in the medium trims it into smaller lengths. Many bacterial species release DNAses into their surroundings, and DNA trimming may be one of their functions. However, it is generally accepted that *N. gonorrhoeae* is not among the bacteria that release a DNAse into the medium since it yields a negative response in the QuadFERM+ test (5) for DNAse activity. Yet, one can demonstrate that colonies of the bacterium, particularly older ones, appear to degrade DNA in their vicinity. You can demonstrate this if you grow them on DNA containing media, such as the agarose formulation described here, and flooding the plate with 1 N HCl to precipitate the DNA. When one does this experiment, the result is a narrow clear zone surrounding the *N. gonorrhoeae* colony indicative of DNA degradation and possible DNAse activity (Data not shown).

The present findings suggest that host proteins may contribute to a hitherto unknown innate resistance mechanism. The fact that lysozyme and serum albumin are able to repress bacterial variation, suggests that this may be occurring in the course of infection. Presumably, these proteins act by sequestering the DNA or in some way prevents the recipient bacteria from importing it. Both proteins are known to form complexes with DNA (7, 13).

The results also suggest a novel approach for intervening in *N. gonorrhoeae* infections. If one can prevent the bacterium from changing either its antigenic façade or into a stress survival variant, the immune system ought to be more efficient at eliminating

it. These experiments indicate that we should be able to inhibit the bacterium's capacity to import the mobile genetic elements in some way, and, thus, decrease the pathogen's capacity to make these changes. This in turn, would provide the immune system an advantage in eliminating the pathogen. There may be several ways of accomplishing this. One could develop a vaccine that elicits antibodies that block the capacity of the bacterium to import DNA fragments. One target antigens might be the DNA methylase since it is required for DNA importation (10). Another might be any DNAase associated with the bacterium. Alternatively, antibodies could target the outer membrane proteins involved with the internalization of the mobile DNA fragments such as the DNA binding domain on the opa protein (4). Yet another approach might be to develop antibiotic-like small molecules that interfere with the bacterium's DNA importation mechanism. While these approaches may not prevent an initial infection, they ought to shorten its duration. Even if the bacterium has multiple mechanisms for antigenic variation, one would expect that abrogating even one, especially one that allows the organism to survive in the face of stress, would provide the immune system with an advantage (12). The assay described here might be a useful tool to discover vaccine antigens as well as small molecules that would reduce DNA transmission. Further, the same strategy for preventing gonococcal variation might be applied to protect us from other mucosal pathogens that survive the host's defenses by the interchange of the mobile genetic elements that lead to antigenic changes.

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Figure Legends

Figure 1. Photograph of a circular gonococcal mat. The outer edge of the mat is comprised of viable cells that continue to expand outward at a slow rate. The dark spots within the mat are foci of viable cells that survive the death of the surrounding cells of the mat. The diameter of the mat is about 2 cm. Because of the possible influence of the conditions near the outer edge of the mat, foci contiguous with the edge were not counted.

Figure 2. Suppression of foci expression by the addition of exogenous agents. No added agent, ■; RNase (0.1 mg/ml), ●; bovine serum albumin (50 μg/ml), ♦; and lysozyme (50 μg/ml), ▲.

Figure 3. Enhanced expression of foci by the addition of DNAse I to the agarose medium. The number of foci within the mats on the DNAse I plates at 120 and 148 hours were too numerous to count. The number of foci seen in the DNAse I (0.1 mg/ml) mats (●) compared to media with no added exogenous agent (■).

Figure 4. Suppression of foci expression by the addition of salmon egg DNA. The number of foci seen in the DNA (0.1 mg/ml) mats (●) compared to media containing no added agent (■).

Figure 1

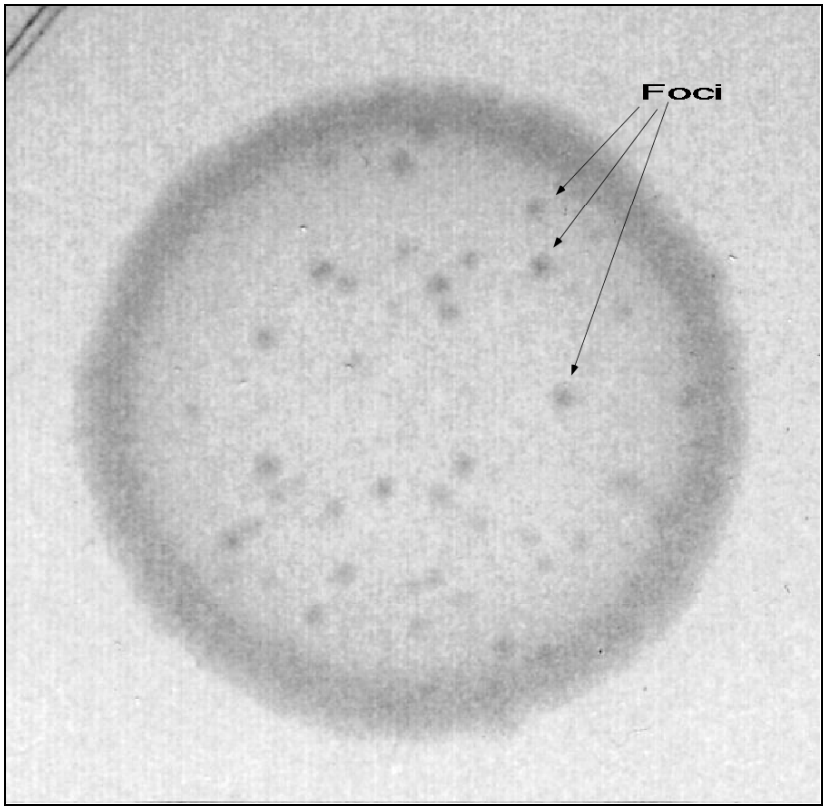

Figure 2

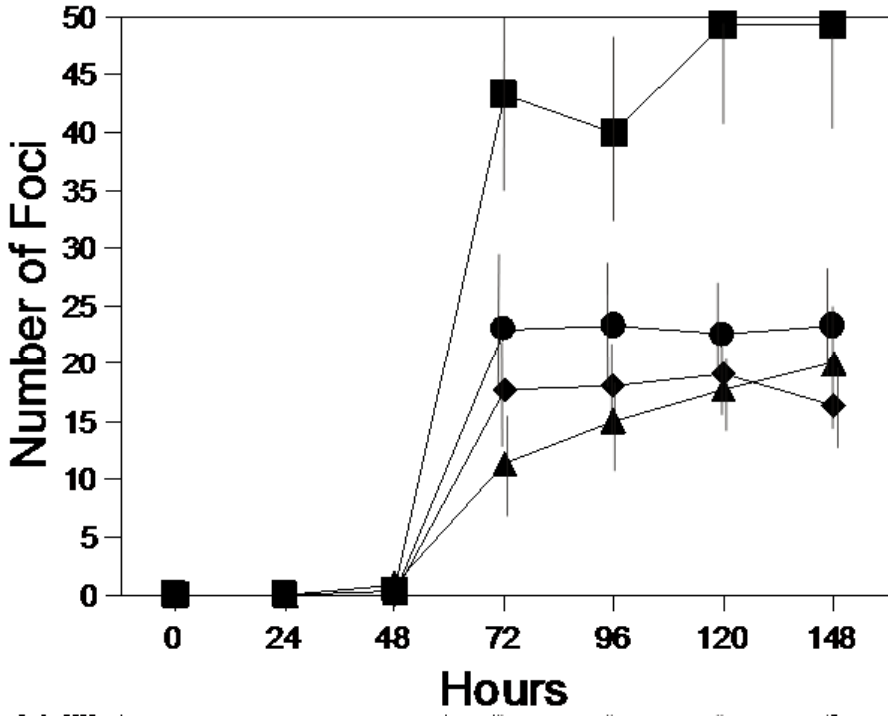
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Figure 3

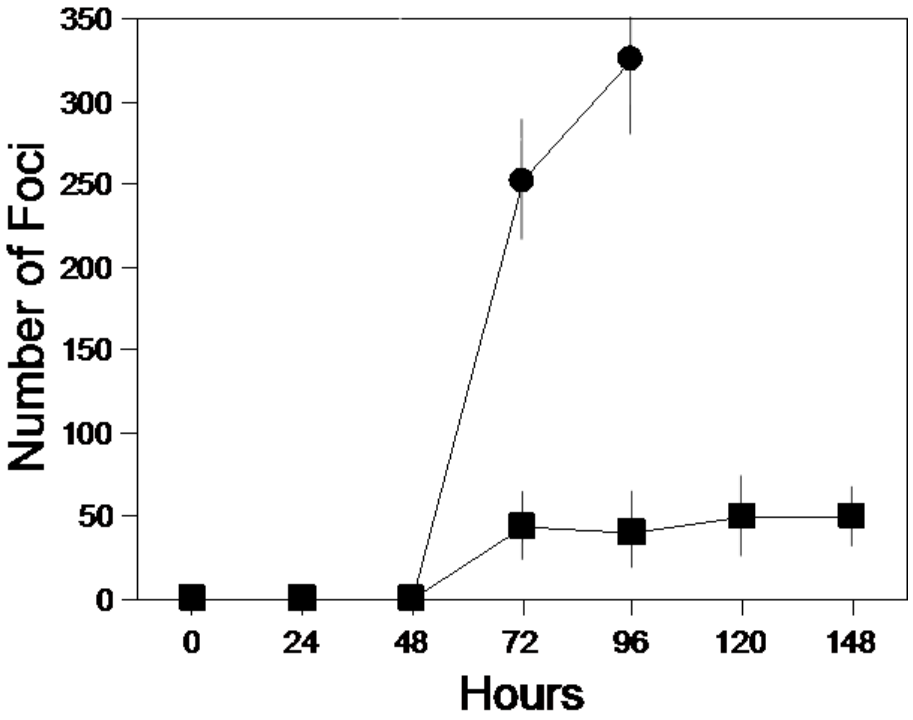
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Figure 4