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Correlation between Precolonization of Trigeminal Ganglia by Attenuated Strains of Pseudorabies Virus and Resistance to Wild-Type Virus Latency†

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We compared the levels of latent pseudorabies virus (PRV) DNA in trigeminal ganglia (TG) of pigs after intranasal inoculation of different PRV strains by using quantitative DNA PCR. The extent of colonization attained in each case varied significantly according to the type of strain and inoculum dose, wild-type (WT) PRV being the most efficient strain in colonizing TG. When groups of pigs representing different levels of precolonization of TG with an attenuated PRV strain were challenged with WT PRV, it became evident that there is a statistically significant inverse correlation between the extent of precolonization attained by an attenuated PRV strain in TG and the level of establishment of latency by superinfec ting WT PRV. The protection against WT PRV latency did not correlate with the extent of WT PRV replication at the portal of entry.

A common characteristic of all members of the subfamily *Alphaherpesvirinae*, including pseudorabies virus (PRV), is the ability to establish latency in sensory neurons following an acute infection (27). In the latent state, the virus does not produce clinical symptoms but it persists indefinitely in the infected individual (9, 25, 27). After specific stimulation, the latent virus can be reactivated and shed to the environment, thus infecting susceptible individuals (1, 9, 15, 18, 25). In some cases, it can also produce recrudescence of clinical disease (9, 25, 27). Latency is, then, very important from an epidemiological standpoint because it is the mechanism by which alphaherpesviruses persist in populations of susceptible individuals (1, 25, 27).

Protection against the latent state of alphaherpesvirus infection has always been a major goal for the development of specific vaccines (15, 26). For PRV, classically attenuated or genetically engineered, modified live vaccines have been used successfully for many years to minimize both clinical symptoms and viral shedding during the acute phase of the infection (33). However, little is known about their influence on the latent phase of the infection (1, 18, 33).

In studies using animal models of herpes simplex virus infections, it has been reported that the establishment of latency in a target tissue by a preceding viral strain precludes the colonization of the same tissue by another strain (3, 10, 16, 32). Epidemiological studies of human infections have suggested that this restriction also exists under natural conditions (13). Subsequently, it was reported that this restriction would not be absolute for herpes simplex virus (11) or bovine herpesvirus 1 (35). Even if a total impairment of wild-type (WT) alphaherpesvirus latency cannot be accomplished, it is possible that the precolonization of latency target tissues by attenuated alphaherpesvirus strains could significantly reduce WT virus latency (9).

We developed a quantitative and differential PRV-specific PCR (29). Using this technique, we show in this report that there is a range of variability among PRV strains in the ability to precolonize trigeminal ganglia (TG) and that a significant correlation exists between the level of latency established by a vaccine strain and the level of protection from latency established by superinfecting WT virus.

For the quantitation of DNA in TG, the entire left and right TG of each animal were combined and processed for total DNA extraction as previously described (5). The quantitative PCR technique has been described elsewhere (29). Briefly, up to 2 μg of DNA, extracted by standard techniques, was amplified in 100 μl of *Taq* polymerase buffer (50 mM KCl, 10 mM Tris - Cl [pH 9], 0.1% Triton X-100, 1.5 mM MgCl) containing 0.200 mM each deoxynucleoside triphosphate, 0.200 mM each PRV-specific primer, 0.800 mM each standard-specific primer, and 5 U of *Taq* polymerase. Amplification was carried out for 40 cycles (each cycle comprised 1 min at 95°C for denaturation, 1 min at 65°C for annealing, and from 3 min [first cycle] to 4 min 20 s [last cycle] at 72°C for extension). After the last cycle, the reaction mixture was further incubated at 72°C for 7 min, to allow the complete extension of all the amplified products.

The targets for amplification, primers, and probe sequences were as follows. For detection of WT virus in pigs infected with both the WT and vaccine strains, the target for amplification was the gene that codes for the nonessential glycoprotein gI. (According to the common nomenclature for alphaherpesvirus glycoproteins agreed upon at the 1993 International Herpesvirus Workshop, the denominations gI, gp63, gX, and gp50, used in this report to name PRV glycoproteins, should be replaced in the future by the denominations gE, gI, gG, and gD, respectively.) Since all the vaccine strains used in the challenge study were gI deleted, these primers would detect and quantitate only superinfecting WT virus. The upstream primer was CCCACGACGAGGACTACTAC, the downstream primer was CGCGGAACCCAGACGTCAAGGC, the amplified sequence was from positions 2673 to 2884 (num-

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were ACGCCCGCCGGCCCT. For detection of vaccine virus in the vaccinated, nonchallenged animals, the target of amplification was the gene that codes for the essential glycoprotein gp50. The upstream primer was CACGGAGGAGCAGCTGG GGCT, the downstream primer was GTCCACGCCGCTT GAAAGCT, the amplified sequence was from positions 434 to 650 (numbered as in reference 19), and the probe for hybridization was CCGCTTGTCGACGGCGGTGAACATC. As the standard used to measure the efficiency of amplification in the different samples, the target of amplification was the gene that codes for the porcine nuclear factor 1 (NF1). The upstream primer was GTCTGCTGCACTCAACCCGG, the downstream primer was CCCGCCTCACCCTGCTCG, the amplified sequence was from positions 900 to 1165 (numbered as in reference 17), and the probe for hybridization was CTC GCCGTGGTGCTCTCCAGCGGGATGC.

The concentration of DNA determined by the quantitative PCR is a relative estimate expressed as viral copies per cell equivalent, assuming that 2 × 10^6 cells contain approximately 1 μg of pig genomic DNA.

The samples that were negative by the quantitative assay were run as a standard PCR, with the amount of input DNA increased. Briefly, up to 5 μg of DNA was amplified under the same conditions as those used for the quantitative assay, but omitting the NFI primers and using only 0.100 mM each PRV primer (specific for either the gI or the gp50 gene) and 2.5 U of Taq polymerase. In this way, the amount of viral DNA can be estimated even when present at very low levels (up to one viral copy in 5 μg of total DNA).

The PCR-amplified products were electrophoresed and analyzed by Southern blot hybridization. The amount of radioactivity hybridized to virus-specific and standard-specific amplified products was evaluated by using an Ambys image analyzer. A titration in which logarithmic dilutions of viral DNA were added to a constant amount of PRV-free pig brain DNA was run in parallel for each experiment. The log of the radioactivity hybridized to the amplified viral target/radioactivity hybridized to the amplified standard ratio was plotted against the log of the number of viral copies per cell equivalent. To calculate the amount of viral DNA in the unknown samples, the same ratio was calculated for the sample and then the number of viral genome copies per cell equivalent was obtained by interpolation of this ratio in the titration curve.

Cross-bred PRV-free pigs (body weight, 40 lbs [18.14 kg]) from a single source, homogenous in genetics, sex, and age, were randomly assigned to treatment groups of 10 animals each. Two experiments were conducted, as described below.

**Experiment 1. Levels of latent DNA in TG attained by different strains.** Experiment 1 was directed towards quantitating the level of colonization that can be established in latency target tissues by different PRV strains administered via the intranasal route, including two attenuated vaccine strains and WT PRV. The four experimental groups, composed of 10 animals each, were as follows. One group was inoculated with 10^5 50% tissue culture infective doses (TCID_{50}) of strain SG (a genetically engineered strain with deletions in the genes that code for glycoprotein gX, glycoprotein gI, and thymidine kinase [phenotype, gI^- gp63^- gX^- TK^-]) (Syntrovet, Lenexa, Kans.). A second group was inoculated with 10^3 TCID_{50} of strain BA (Bartha strain, a conventionally attenuated strain with a deletion of the glycoprotein gI and gp63 genes [phenotype, gI^- gp63^- gX^- TK^-]) (Bio-Ceutic Laboratories, St. Joseph, Mo.), while the pigs in a third group received 10^7 TCID_{50} of strain BA. Finally, a fourth group consisted of 15 animals that were inoculated with 10^5 TCID_{50} of WT PRV (Becker strain) (obtained from K. Platt, College of Veterinary Medicine, Iowa State University, Ames) by intranasal inoculation and eye instillation. Each group was kept in a separate isolation room, under strict biocontainment management. Only the animals inoculated with WT PRV (Becker strain) exhibited frank clinical disease, and four of them died with clear symptoms of pseudorabies during the acute period of the infection. Ten of the 11 surviving animals were randomly selected for quantitative evaluation of PRV DNA. The pigs in the four groups were killed 37 to 43 days postinfection, and the TG were collected and immediately frozen at −70°C.

The efficiency of colonization was found to be both dose and strain dependent. As shown in Fig. 1, the tested vaccine strains were less efficient in colonizing TG than was the WT PRV Becker strain, which was able to establish colonization above 10^7.18 viral copies per cell equivalent (1 viral genome copy every 400 cell equivalents) in all of the pigs (Fig. 1), in agreement with values previously reported by others (28). With strain BA applied at a higher dose (10^7 TCID_{50}), the mean colonization level was 10^{-2.86} viral copies per cell equivalent (1 viral genome copy per 760 cell equivalents), with 5 of 10 animals demonstrating levels of latency above 10^{-5.39} viral copies per cell equivalent (approximately 1 viral genome copy per 2,500 cell equivalents). When strain BA was applied at a low dose (10^5 TCID_{50}), the mean level of latent DNA was 10^{-4.28} (approximately 1 viral genome copy per 1.7 × 10^4 cell equivalents), with 8 of 10 animals showing levels of latency that ranged from 10^{-3.39} viral copies per cell equivalent (approximately 1 viral genome per 2,500 cell equivalents) to 10^{-5.0} viral copies per cell equivalent (1 viral genome copy every 10^2 cell equivalents) (Fig. 1). Finally, strain SG attained a mean colonization level of 10^{-5.21} (approximately 1 viral genome copy per 1.5 × 10^2 cell equivalents), with 9 of 10 animals...
exhibiting levels of latency below $10^{-5.0}$ viral copies per cell equivalent (1 viral genome copy every $10^5$ cell equivalents). SG was, then, the strain that established the lowest levels of latency of all the groups. It is important to note that for any given viral strain, the level of colonization varied widely among different animals (i.e., a range of 2 log units for strain BA at either a high or a low dose) (Fig. 1), which is consistent with the significant individual variability in latency levels established by attenuated PRV strains that we reported previously (29).

**Experiment 2. Challenge with WT PRV.** In experiment 2, the objective was to ascertain how precolonization of latency target tissues by an attenuated vaccine quantitatively affects the establishment of latency by superinfecting WT PRV. We established three experimental groups ($n = 10$ each) representing the three previously determined (experiment 1) different levels (low, medium, and high) of precolonization of TG by an attenuated vaccine strain. Therefore, one group of pigs was inoculated with $10^5$ TCID$_{50}$ of strain SG, a second group of pigs was inoculated with $10^3$ TCID$_{50}$ of strain BA, while a third group was inoculated with $10^2$ TCID$_{50}$ of strain BA. Forty-five days later, a serum sample was drawn from each animal and each group was challenged with $10^5$ TCID$_{50}$ of PRV (Becker strain) by simultaneous intranasal and conjunctival inoculation. WT PRV clearance was evaluated by periodic sampling (swabs taken every 2 days from day 3 until day 11 postchallenge) of pharyngeal and conjunctival mucosae of all the challenged animals in the three groups. Infectious virus present in these samples was titrated by standard microtitration methods by using Reed and Muench interpolation. Clinical observation was performed daily. The three groups were killed 37 to 40 days after challenge (about 85 days after vaccination), serum samples were collected at the time of killing, and the TG samples were processed as described for the groups used in the first experiment.

Figure 2 shows the frequency distribution of the levels of latency attained by the challenging WT virus for each group, compared, in each case, with the levels of precolonization attained by the vaccine strain and doses used for each group as determined in the previous experiment (Fig. 1). A statistically significant inverse correlation (Pearson’s $r = -0.577$; $P < 0.0001$) between the levels of latency attained by each of the precolonizing vaccine and WT strains was found for the three groups. In 6 of the 10 pigs vaccinated with $10^5$ TCID$_{50}$ of the Bartha strain (the best-colonizing strain), no WT viral DNA could be detected (limit of sensitivity, approximately one viral genome per $5 \times 10^6$ cell equivalents) (Fig. 2A). Furthermore,
TABLE 1. Geometric means of TG precolonization levels attained by three PRV inoculations and mean level of WT PRV colonization established upon challenge of the three precolonized groups

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean precolonization by preceding strain (SE)</th>
<th>Mean WT PRV colonization after challenge (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PRV</td>
<td>NA$^a$</td>
<td>&gt;1.80$^b$</td>
</tr>
<tr>
<td>SG</td>
<td>−5.21 (0.190)</td>
<td>−1.777 (0.286)</td>
</tr>
<tr>
<td>BA (10$^5$)</td>
<td>−4.236 (0.257)</td>
<td>−2.799 (0.471)</td>
</tr>
<tr>
<td>BA (10$^7$)</td>
<td>−2.88 (0.464)</td>
<td>−4.281 (0.633)</td>
</tr>
</tbody>
</table>

$^a$ NA, not applicable.

$^b$ Values are expressed as logs of the number of viral copies per cell equivalent.

TABLE 2. Geometric means of neutralizing antibody titers (twofold dilutions) after precolonization and at necropsy of groups shown in Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>SN titer 45 days after precolonization (SE)</th>
<th>SN titer at necropsy (37-45 days postchallenge) (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG</td>
<td>2.1 (1.25)$^a$</td>
<td>87.24 (27.6)</td>
</tr>
<tr>
<td>BA (10$^5$)</td>
<td>6.5 (4.63)$^a$</td>
<td>142.2 (89.6)</td>
</tr>
<tr>
<td>BA (10$^7$)</td>
<td>9.0 (9.50)</td>
<td>152.9 (105.5)</td>
</tr>
</tbody>
</table>

$^a$ ANOVA indicated no statistically significant differences among the three groups ($F_{2,21} = 2.566; P > 0.1$).

The shedding of WT PRV after challenge of the three differently precolonized groups was quantitated by taking conjunctival and pharyngeal swab samples of all challenged animals at 3, 5, 7, 9, and 11 days postchallenge. As shown in Fig. 3, there were no statistically significant differences (Fisher’s test [31]) in either the amounts or the lengths of time of viral shedding among the three treatment groups. In all cases, differences within each group were larger than those between groups. Daily clinical evaluation of the animals indicated no significant clinical differences among the three groups following the challenge. All of the animals in the three groups were well protected in comparison with the nonvaccinated group that received an identical dose of WT PRV (Becker strain) (group IV, experiment 1) (Fig. 1), in which most of the animals became severely ill, with several of them (4 of 15) dying.

The results of quantitative PCR for PRV DNA clearly reflect individual-to-individual variability in the level of latency established by PRV strains. However, the quantitative PCR allowed us to group and compare strains and, furthermore, to establish correlations between the strains’ distinct abilities to colonize target tissues. The results of the challenge of precolonized groups with WT PRV provide the most significant conclusion of this report: the higher the frequency of precolonization by a preceding (attenuated) strain in TG of one particular group of animals, the lower the extent of latent WT PRV colonization established in these tissues following superinfection.

One apparent way to explain the differences in WT PRV colonization observed among the three groups would be based on differences in the extent of peripheral replication of WT PRV at the portal of entry and therefore more or less seeding of WT PRV at the TG level. However, when we compare the levels of shedding of WT PRV at the mucosal-surface level, no significant difference in either the length or intensity of the clearance period could be noticed among the groups (Fig. 3), indicating that similar levels of peripheral replication of WT PRV took place in all cases. The data on neutralizing antibody levels indicate that in only one group (Table 2, SG group) was the level of circulating antibodies established by the precolonization lower than in one of the other two groups (BA, 10$^5$ TCID$_{50}$). This discrepancy between circulating antibody levels and certain parameters of clinical protection against herpesvirus infections, such as viral shedding, has been well documented previously (8, 30). Interestingly, one of the six pigs that...
FIG. 3. WT PRV shedding upon challenge. Viral shedding by the animals vaccinated with strain BA at $10^7$ TCID$_{50}$ (asterisks), strain BA at $10^5$ TCID$_{50}$ (open circles), and strain SG at $10^5$ TCID$_{50}$ (closed circles) is shown. Pharyngeal (A) and conjunctival (B) swabs were taken and processed as described in the text. Each point is the geometric mean for 10 pigs in each group. Standard deviations for all time points for all groups were between 0.5 and 2.2 log units. Differences among treatment groups are not statistically significant (Fisher's test) (31).

were completely protected from WT latency (Fig. 2A; group BA, $10^7$ TCID$_{50}$) was the one that shed the largest amount of virus after challenge in this group. Furthermore, in analyzing animal-by-animal viral shedding and the corresponding level of latency established by the superinfecting WT PRV, no correlation was evident between these two parameters ($r = -0.1359; P = 0.503$) (Fig. 4). In addition, we did not observe significant clinical differences among the three groups during the early postchallenge period, as all of the animals from the three groups were fairly well protected. Therefore, although a direct immunological modulation of the establishment of WT PRV latency cannot be ruled out by these experiments, impairment of replication at the portal of entry does not seem to explain the differences observed in levels of colonization of TG by WT PRV.

If the differences in establishment of WT PRV latency are not based on differences in WT PRV replication at the primary site of replication, it is conceivable that the blocking of WT PRV establishment takes place at the translocation (axonal path) or neuronal level instead. The protection against WT latency observed in our experiments could be an example of resistance at the level of the tissues that are the target for latency, for which preinfection of the neurons of that tissue with another (preceding) strain of PRV is required. The case of ganglionic resistance we observed would then be a special example of intracellular interference active against homologous or homotypic viruses (24). It is possible that the interference is based on some of the viral functions (especially LAT gene activity) expressed by the interfering strain during latency or reactivation (4, 22, 23, 25) or on some competition for certain important cellular factors that would be essential to the establishment and/or maintenance of neuronal latent infection (12). The apparent occurrence of a certain threshold for latency (which could be defined by the fact that the observed frequency of establishment of latency above $10^{-3}$ viral copies per cell equivalent [1 viral genome copy per 1,000 cells] for a given vaccine would be somewhat equivalent to the frequency

FIG. 4. Individual WT PRV infectivity titers in pharyngeal samples upon challenge versus latency levels attained by WT PRV. Mean infectivity titers of the samples of pharyngeal swabs are plotted against the latency levels attained by WT PRV in the TG of each animal from the three WT-challenged groups.
of protection of challenged animals from the establishment of WT PRV latency [Fig. 1 and 2]) would be consistent with the models that propose that only a subset of TG neurons are capable of harboring a latency infection (14). Under these conditions, once this susceptible subset of cells has been latently infected by the preceding vaccine strain, they cannot be superinfected with the WT challenging virus. Another plausible explanation for the interference with WT PRV colonization could be based on direct involvement of the immune response at the level of the site of colonization, in this case, the TG. Further studies should certainly be directed towards addressing these points.

In this study, we have found that there is a significant inverse correlation between the level of latency established by an alphaherpesvirus live modified vaccine and the level of latency established by a superinfecting WT virus in the natural host of the infection. Therefore, modified live virus vaccines administered through routes of immunization that maximize coloniza-
tion of latency target tissues may significantly decrease latency of WT strains. The different genetic deletions of the strains which we used have certainly influenced the different coloniza-
tion abilities observed in our experiments. It has been reported that PRV gI is an important factor in targeting this virus to the peripheral nervous system (2, 34) and also that thymidine kinase is important for herpesvirus replication in quiescent cells (i.e., neurons) (7, 21). The simultaneous dele-
tion of the genes encoding both these factors in strain SG should then have impaired both targeting of the virus to neurons and viral replication in neurons, therefore explaining the poor colonization of TG observed with this strain. It is interesting that colonization studies conducted in our labora-
tory with a gI+ strain isogenic to SG (strain SMB; Syntrivet) (data not shown) showed that this strain has colonization ability significantly greater than that of any of the strain-dose combinations reported in this paper, thus not only confirming the important role of PRV gI in targeting neural tissue but also suggesting that gI+ attenuated PRV strains may have optimal ability to precolonize TG and therefore to interfere with superinfecting WT latency. Considering the results herein reported, the ability of a vaccine to establish latency by itself would then be a subject worth pursuing.

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