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Co-Infection of the Rat Central Nervous System with Genetically Engineered Strains of Pseudorabies Virus

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유전자 조작된 Pseudorabies 바이러스에 의한 흰쥐 중추신경계의 이중감염

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<국문초록>

중추신경계의 미주신경등쪽핵(DMV)내 유사핵분열후 신경세포로 외래 유전자를 전달하는 매개체로서 pseudorabies 바이러스(PRV)의 유전자 조작기술은 흰쥐의 결장내로 PRV를 주입시킨 후 복잡한 신경로 추적에 관한 연구에서 하나의 바이러스에 의해 얻어지는 것보다 더욱 유용한 결과산출이 가능하게 하였다. 본 연구에서는 흰쥐의 생체내 실험모델로 하나의 바이러스 또는 이중 바이러스 주입에 PRV의 유전자 조작된 2 종의 바이러스를 사용하였다. 이 2 종의 바이러스는 PRV의 Bartha 종에서 유래되었지만 면역조직화학적으로 검출할 수 있는 동일한 유전산물을 산출할 수 있도록 구성되었다. PRV-BaBlu는 PRV 게놈의 Us 구역 중 gC 자리에 lacZ 유전자를 삽입하여 산출되었는데 β-galactosidase 발현은 이 바이러스에 감염된 신경원의 독특한 표시자로 나타났다. PRV-D는 2가지 단계에 의해 조성되었는데 첫째, PRV-Bartha의 Us 구역의 일부 유전자를 제거하고, 야생형인 PRV-Be DNA로 복구시켰는데 이로써 PRV-D는 PRV-Bartha 또는 PRV-Bablu에 존재하고 있지 않는 외피 당단백질인 gE와 gI를 지니게 되었다. 본 연구의 결과는 다음과 같았다.

첫째, PRV-D의 개별적 접종에 의해 얻어진 감염은 PRV-BaBlu에 의한 동일 신경회로의 감염보다 유의하게 빨랐다.

둘째, 유전자 조작된 PRV의 변이종은 변이종 상호간 및 부모 바이러스와 상이하였다. PRV-D는 PRV-Bartha 또는 PRV-BaBlu보다 감염독성이 더 강했고, PRV-BaBlu는 PRV-Bartha보다 감염독성이 약했다.

셋째, 결장을 지배하는 미주신경등쪽핵내 신경원은 변이종 바이러스들을 동시에 접종하였을 경우 이중감염을 나타내었다.

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I. INTRODUCTION

PRV is very useful tool to investigate the neuronal tracing research owing to its neurotropism and transsynaptic power(김진상 등, 1997a; 김진상 등, 1997b; 김진상, 1998; 이성준 등, 1997; Card, 1995; Card et al., 1998; Enquist et al, 1999; Kristensson et al., 1996). Efforts to further exploit the invasiveness of PRV to define populations of functionally related neurons have also taken advantage of genetically engineered strains of virus that express unique gene products(Jansen et al, 1995; Levatt et al, 1998). In a notable application of this experimental approach, Jansen and colleagues(1995) injected two genetically modified forms of the Bartha strain of pseudorabies virus(PRV-Bartha) into different peripheral targets. CNS neurons replicating both strains of virus were demonstrated in some of the experimental animal (Barnet et al, 1993; Card et al., 1995; Card et al., 1991), but the percentage of animals that exhibited coinfected neurons was remarkably small. In order to investigate the coinfection of two PRV mutants in single neuron in DMV innervating rat colon, we used PRV-D and PRV-Bartha. One of the mutants had a titer of 104.5 plaque forming units per milliliter(pfu/ml) and the other was approximately 107 pfu/ml. Given the fact that prior studies with PRV and Herves Simplex Virus(HSV) have demonstrated that the percentage of animals that developed a productive infection drops substantially when less than 105 pfu was injected (Card et al, 1995; Sun et al, 1996; Ugolini et al, 1987) it is not surprising that the percentage of animals exhibiting dual infection in the Jansen study was small.

We have tested the hypothesis that virulence and rate of replication may influence the ability of two engineered strains of PRV to co-infect neurons in DMV innervating rat in vivo. This hypothesis is based upon the demonstrations of superinfection exclusion with HSV(Campadelli-Fiume et al, 1988, 1990; Johnson & Spear, 1989). To address this hypothesis we examined the infectivity of two genetically engineered strains of PRV-Bartha engineered to express unique proteins. The invasiveness of these strains in single and dual injection paradigms into rat colon was analyzed in central circuitry previously subjected to rigorous analysis with the parental strain of virus.

Ⅱ. Materials and Methods

1. Experimental Animals

Adult male Sprague-Dawley rats(n=20) weighing 180-290 gram at the time of inoculation were used in this investigation. All inoculations were done in a laboratory approved for use of class 2(BSL-2) infectious agents and the animals were housed in this facility throughout the balance of the experiment.

2. Viral Strains

1) PRV-Bartha, an attenuated vaccine strain, served as the parental strain for both of the mutants used in this investigation(Bartha, 1961). The virulent wild type strains PRV-Becker(Becker, 1967) and PRV-Ka were used in studies of viral virulence (PRV-Becker) and for construction of one of the mutants (PRV-Ka).

2) PRV-BaBlu contains the lacZ gene in the gG locus of the unique short (Us) region of the viral genome and expresses β-galactosidase (β-Gal) under the control of the gG promoter. Construction of this mutant followed the methods of Mettenleiter and Raugh(1990) and has been described in a prior analysis of cardiac circuitry(Standish et al, 1995). Briefly, a lacZ expression cassette of the gG promoter and the first seven codons of PRV-Becker gG fused to lacZ was crossed onto the gG locus of the PRV-Bartha genome by homologous recombination. The genome structure of the plaque purified recombinant was verified by restriction enzyme analysis and

Southern blotting. PRV-D was constructed in two steps by Tamar Ben-Porat and the invasivenes of this mutant in visual circuitry has been described(Card et al, 1992). To produce this strain the Us deletion of PRV-Bartha was first repaired with DNA from PRV-Ka to restore the gE and gI genes. The gE gene was then selectively deleted, a manipulation that reduces the virulence of this strain(Card et al, 1992; Whealy et al, 1993). The gI gene, which is present in PRV-D, but is absent in PRV-BaBlu, provides the unique marker of neurons infected with this virus.

All strains of virus were grown to a minimum titer of 108pfu/ml in PK-15 cells. Specific titers in pfu/ml were : PRV-Becker=5.5×108, PRV-Bartha=6.25×108 , PRV-D=2.5×108, PRV-BaBlu=4.75×108. Each virus stock was aliquoted at 50-100μl/tube and stored frozen at -80°C. Aliquots were thawed immediately prior to injection and unused portions were inactivated with Clorox and discarded(Flg.A).

3. Antisera.

The following reagents were used to localize infected neurons in DVM innervating rat colon. A rabbit polyclonal antisera (Rb133) generated against acetone inactivated virus recognized all strains of virus used in this analysis (Card et al, 1990). PRV-D infected cells were identified with a polyvalent rabbit antiserum (Rb1544) that recognizes the glycosylated precursor of gI(formerly designated gp63 in older PRV literature). This antibody was generated by immunizing rabbits with a polypeptide corresponding to amino acids 60-268 of gI expressed in Escherichia coli. PRV-BaBlu was identified with mouse monoclonal antibodies generated against β-Gal purchased from Sigma Chemical Company and 5 Prime-3 Prime, Inc(Bolder,CO).

4. Experimental Rationale

Two well charaterized models of the invasiveness of PRV were used in this analysis. Each model has the advantage of peripheral inoculation and addresses different aspects of viral invasiveness and cell to cell transmission. Primary invasiveness of neuronal perikarya and anterograde transneuronal infection of the CNS was examined by injection of virus into proximal and distal colon. This route of inoculation causes an initial infection of DVM neurons cells followed by anterograde transneuronal infection of

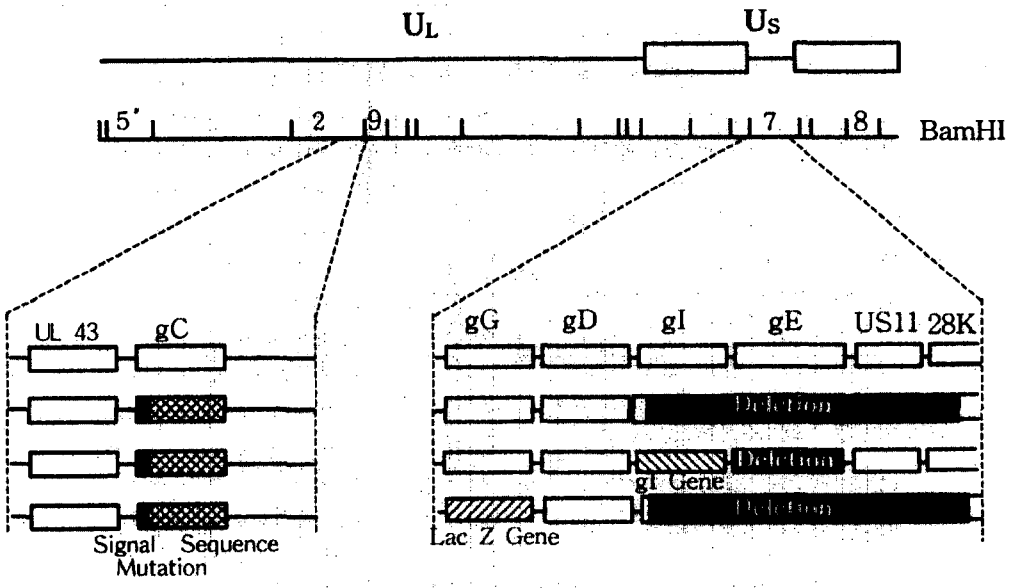

Fig A. The genetic map of pseudorabies virus (PRV).

neurons in the diencephalon and midbrain.

All animals were anesthetized with ketamine and xylazine injected intraperitoneally prior to any experimental manipulation. Rats included in the analysis of visceral movement received an injection of one or both viruses in single or dual injection paradigms into rat colon. In single injection experiments, the invasiveness of each strain was evaluated by injection of 4 μl of viruses into the proximal and distal colon or simultaneous injection of the viruses into different colon sites. Three dual injection paradigms were used. In the first, a mixture of PRV-D and PRV-BaBlu(2μl of each) was injected into the colon as described previously. The second paradigm involved injection of 4μl of PRV-BaBlu into colon followed by injection of an equivalent volume of PRV-D into the same area 24 hours later. The third paradigm reversed the order of virus injection used in the second paradigm. All animals were sacrificed 48~140 hours after the initial inoculation and the brain was processed for immunohistochemical localization of infected neurons as described in the next section.

5. Tissue Processing

Animals were anesthetized with an overdose of ketamine/xylazine and killed by transcardiac infusion of buffered aldehyde solutions. The brain was removed and postfixed for one hour at 4°C prior to cryoprotection in 20% phosphate buffered sucrose at the same temperature and fixative. Tissue was then cut in the coronal plane at 35 μm per section using a freezing microtome and stored in cryopreservant (Watson et al, 1986) at -20°C prior to immunohistochemical analysis. Immunohistochemical localizations were conducted using immunoperoxidase or immunofluorescense procedures. In both instances, tissue was first washed to remove cryopreservant and then transferred to primary antibodies for a 24~48 hour incubation at 4° C. The immunoperoxidase localizations were accomplished with the avidin-biotin modification(Hsu et al, 1981) of the peroxidase-antiperoxidase method using affinity purified secondary antibodies and Vectastain reagents. The immunofluorescence localizations used secondary antibodies conjugated to fluoroscein isothiocyanate(FITC), CY2 or CY3 to produce green (FITC, CY2) or red (CY3) fluorescence. These fluorescent secondaries were used at a dilution of 1 : 1000. Processed sections from tissue processed with these procedures were coverslipped with Fluoromount G prior to being analyed and photographed with a Olympus B201 photomicroscope. Tissue processed with the CY2 and CY3 conjugated secondary antibodies were dehydrated, cleared and coverslipped with Cytoweal 60.

6. Experimental Analysis

Virulence of each strain was determined by documenting symptoms of viral infection in all animals. We did not conduct a systematic analysis of mean time to death. However, a subset of the longest surviving animals infected with each strain succumbed to infection and we carefully monitored the animals for overt signs of viral infection(lethargy, weight loss, spiked coat).

The extent of infection in the DMV produced by each strain of virus was initially assessed in tissue processed for immunoperoxidase localization of viral antigens with the rabbit anti-PRV polyclonal antiserum that recognizes all strains of PRV used in this study. Immunoperoxidase localizations were then conducted on tissue from each animal using the gI and β-Gal monoclonals. Co-infection of neurons in cases receiving dual inoculations was determined in dual labeling immunofluorescence localizations. The gI glycotrotein was identified with the secondary conjugated to FITC or CY2 to produce green fluorescence and β-Gal was identified with the CY3 conjugated secondary to produce red fluorescence. Fluorophors in each sample were excited with the

appropriate filters and infected neurons were photographed.

Ⅲ. Results

The patterns of infection produced by the parental virus in colon innervating circuit were also observed following injection of the engineered PRV mutants. However, differences in virulence and the progression of infection through autonomic circuitry were identified. Analysis of the efficiency of co-infection of neurons in colon circuit provided compelling evidence that the differences in virulence and invasiveness of the two engineered mutants effected their ability to efficiently replicate in the same neurons.

1. Engineered PRV mutants differed in virulence.

In single injection paradigms, PRV-BaBlu was less virulent than both PRV-Bartha and PRV-D. In contrast, PRV-D was more virulent than PRV-Bartha and PRV-BaBlu. These determinations were based upon the appearance of symptoms of infection (lethargy, spiked coat, oral and nasal secretions) and the time of death of a limited number of animals that succumbed to infection.

These data suggest that the adverse effects of PRV-D infection derive largely from infection of peripheral organ systems rather than infection of the brain.

Animals included in co-injection paradigms involving simultaneous or temporally separated injections of PRV-D or PRV-BaBlu displayed virulence similar to that produced by individual inoculation of PRV-D. In this investigation the virulence observed after simultaneous inoculation of the two strains was similar to that produced by the more virulent PRV-D virus in single injection paradigms. This increased virulence occurred even though the amounnt of virus injection in the co-injection paradigm was approximately the same as was injected in the single injection paradigms. PRV-D also dictated the virulence when injection of the two strains was temporally separated by 24 hours. As one might predict, the magnitude of virulence in the co-injection studies depended upon the sequence of injection of the two strains. That is, virulence was greater when injection of PRV-D preceded injection of PRV-BaBlu. Collectively, these data indicate that virulence in co-injection paradigms will be determined by the more virulent strain of virus.

2. The progression of transneuronal infection differed in animals infected with genetically modified strains of PRV.

Comparison of the temporal progression of infection through colon innervating circuitry following infection of animals with PRV-D and PRV-BaBlu revealed dramatic differences in the extent of transynaptic passage of virus compared to one another and the parental virus. Restoration of the gI gene to the Us region of the PRV genome substantially increased the rate of viral replication and/or transport through circuit and, as shown above, also increased the virulence of infection. In contrast, substitution of the lacZ gene for gG in PRV-BaBlu reduced the progression of infection through each circuit. Thus, direction of viral transport into the nervous system(anterograde versus retrograde) had no apparent influence upon the differences in invasiveness. PRV-BaBlu and PRV-D also displayed differences in the retrograde progression of infection through autonomic circuitry innervating the colon.

Collectively, these data demonstrate that the progression of infection produced by PRV-D exceeds that produced by PRV-BaBlu in the same circuitry by approximately 24 hours.

Furthermore, the data demonstrate that this difference in the temporal progression of infection occurs irrespective of the direction of viral transport into the nervous system. Co-infection of neurons with

PRV-BaBlu and PRV-D was observed in this model. However, the ability of both strains to replicate in the same neurons in DMV was dependent upon the simultaneous exposure of permissive neurons to both viruses. If temporal differences occurred in the invasion of one strain of PRV, it was not uncommon for the neuron to become refractile to replication of the second strain. Thus, the temporal presentation of the two strains of virus to first-order neurons was extremely important in determining rates of co-infection.

It is important to recognize that the ability to interpret the coinfection data is limited by the increased virulence that occurs in the dual inoculation paradigms. For example, the increased virulence resulting from dual inoculation makes it difficult to evaluate the ability of PRV-BaBlu to establish an infection because of the slower rate of neuroinvasiveness that is characteristic of this strain. Therefore, we were able to determine the efficiency of anterograde transynaptic infection in circumstances in which the two viruses did not have to compete with one another for the initial invasive event. Considerated together, these data sets provide important insights into the ability of two strains of virus to coinfect neurons in vivo.

The progression of infection observed following simultaneous injection of a mixture of both viruses into the wall of colon reproduced that demonstrated in single injection paradigms. That is, PRV-D infected a larger population of neurons earlier in the course of infection than PRV-BaBlu. This was reflected by the presence of the unique marker of this virus by post inoculation. These neurons also exhibited β-Gal immunoreactivity, indicating that they were replicating both strains of virus, and with advancing survival, but the relative percentage of neurons infected with one or both strains of virus remained the same. This is apparent in comparing the extent of PRV-BaBlu infection at 80 hours after individual inoculation with that produced in the dual inoculation paradigm at 9 hours. These data are similar to those observed in the eye model in that they suggest that the more rapid replication and invasion of PRV-D makes DMV neurons more resistant to replication of PRV-BaBlu. However, the data also suggest that the opposite is not true. The nucleus and cytoplasm of many with the important exception that there were more neurons that contained β-Gal immunoreactivity. However, the magnitude of PRV-BaBlu replication in the DMV in this co-injection paradigm still did not approach that achieved at an equivalent post inoculation interval after individual inoculation with this virus did not compromise the ability of PRV-D to replicate in the DMV and pass were observed in the paraventricular nucleus (PVN) that contained.

The conclusions that could be drawn from reversing the temporal sequence of injention (PRV-D followed 24 hours later by PRV-BaBlu) were substantially limited by the added virulence of the co-injection paradigm and the slower invasion of PRV-BaBlu. The subsequent inoculation of PRV-BaBlu did not alter the temporal dynamics of PRV-D invasion under these circumstances. No PRV-BaBlu infected neurons were observed in these animals, a finding that is not surprising given the fact that the postinoculation interval following injection of PRV-BaBlu was only 48 hours. One longer-surviving animal in this paradigm died unexpectedly at approximately 84 hours. Since this short survival limited the information that we might obtain on PRV-BaBlu replication we did not prusue this experiment any further(Fig.B).

Ⅳ. Discussion

Superinfection exclusion is phenomenon that is well documented in vitro but has not been systematically examined in vivo. Characterization of this process has largely emerged from in vitro studies of HSV-1 infectivity which focused upon the gD envelope glycoprotein. Campadelli-Fiume and colleagues(1998,

1990) demonstrated that gD expressing cell lines are resistant to infection by HSV-1, providing evidence in support of the earlier hypothesis advanced by Tognon and coworkers (1981) that a "virus function" precludes reinfection of cells by progeny virus. Johnson and Spear (1989) further demonstrated that the ability of gD to prevent reinfection was influenced by the amount of gD expressed, the concentration and serotype of the challenge virus, as well as properties of the target cells. These compelling observations have important implications for the life cycle of the virus in that they provide a mechanism that protects against the reduction in infectious yield that would result from reinfection of parent cells by progeny virus. In the present study we provide evidence that exclusion also influences the ability of more than one strain of pseudorabies virus to coinfect neurons in vivo. The data demonstrate that prior infection of neurons with one strain of PRV can render neurons resistant to replication of a second strain of this virus. Importantly, the data also reveal that the outcome of co-inoculation is heavily influenced by the virulence of the infecting strains of PRV. These data provide further insights into the influence of superinfection exclusion upon the spread of PRV through the nervous system and also demonstrate principles important for interpreting data derived from studies in which multiple engineered strains of virus are used to define the organization of neuronal circuitry.

Available data supports the conclusion that the difference in virulence of the two PRV strains is related to the presence or absence of envelope glycoprotein gens in the Us region of the PRV genome. Prior analysis of PRV has shown that deletion of one or both of the gE and gI envelope glycoproteins from this region of the wild type genome reduces the virulence produced by intravitreal inoculation of wild type PRV(Card et al, 1992; Whealy et al, 1993). Similarly, recent work has demonstrated that selective deletions of the carboxy terminus of gE produce a similar reduction of virulence but do not compromise viral invasiveness of visual or autonomic circuits(Tirabassi et al, 1997; Yang et al, 1999). The current findings are similar in that they demonstrate reduced virulence in strains of PRV after deletion of Us genes. However, the alterations of the viral genome in PRV-BaBlu and PRV-D produced strains of virus that were distinctly different in virulence, both from one another and the parental virus. Substitution of lacZ for gG in PRV-BaBlu further attenuated the virulence of PRV-Bartha and also slowed the invasiveness of the virus. To our knowledge this is the first demonstration of a phenotype for gG. In contrast, PRV-D, a virus in which gE is absent but gI was restored by homologous recombination, exhibited increased virulence and invaded both circuits more rapidly than the parental virus or PRV-BaBlu.

Earlier in vitro studies of superinfection exclusion revealed that gD expressing cell lines refractile to HSV-1 replication maintained the capability to endocytose viral particles(Campadelli-Fiume et al, 1988, 1990). This and other evidence indicates that the endocytosed viral particles are fated for destruction in lysosomal compartments and that the blockade of productive replication is due to interference of membrane bound gD with fusion of viral envelopes with the plasma membrane of permissive cells. Our data suggest that exclusion of the replication of a second strain of PRV can also occur intracellularly, after both strains of virus have entered a permissive cell through fusion-based invasion. The clearest evidence in support of this conclusion was provided by the findings of experiments in which both viruses were injected into the same site, either simultaneously or in temporally separated injections. The demonstration of simultaneous replication of both strains of PRV by some neurons clearly demonstrated that, under the proper circumstances, two strains of PRV can successfully invade the same neuron through a fusion-based route. However, the data also revealed that prior infection of a neuron by PRV-D could preclude replication of PRV-D. This result implies that a viral protein encoded by PRV-D, but lacking in PRV-

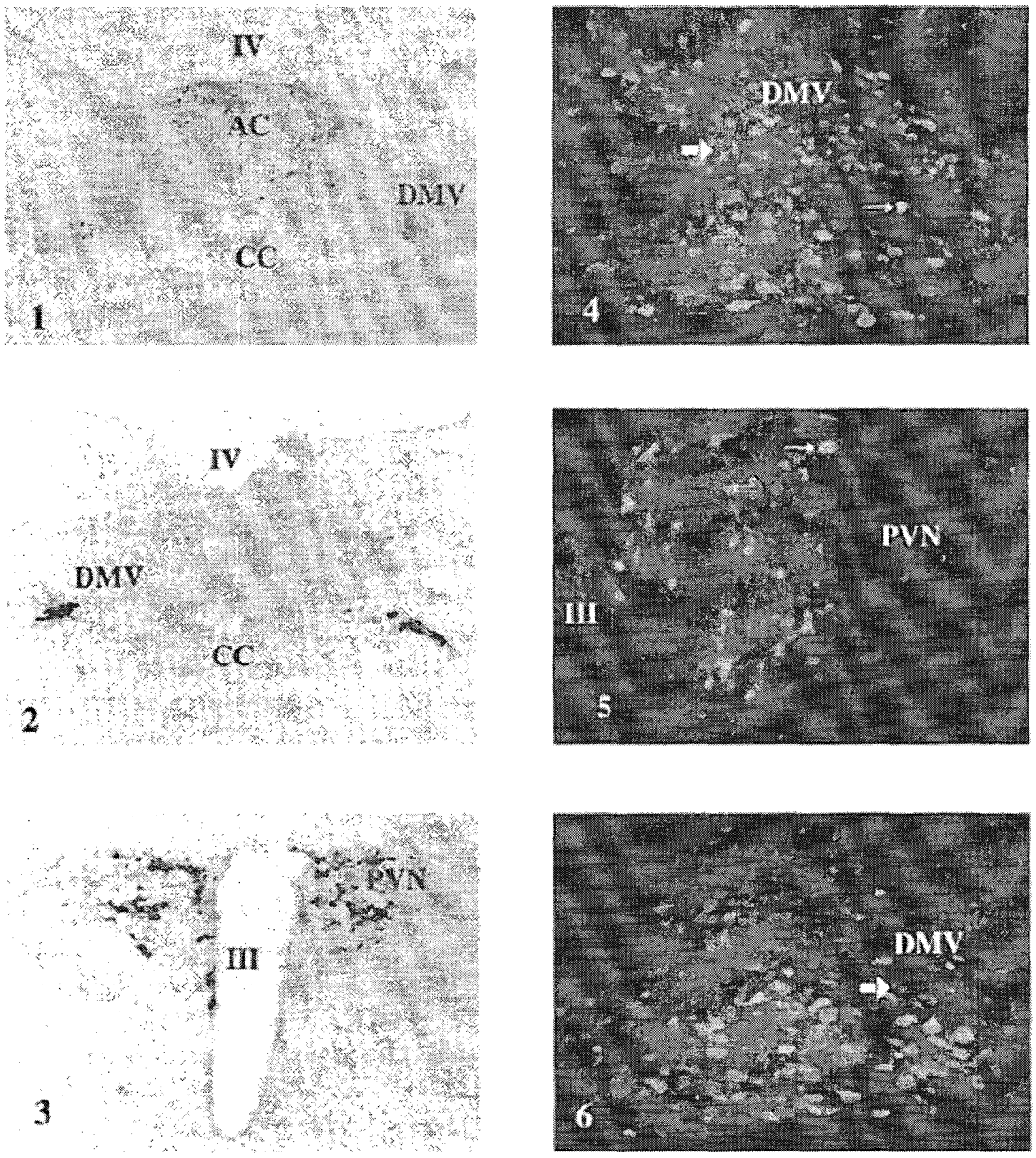

Fig B. ***The microscopic photographs of paraventricular nucelus (PVN) and dorsal motor nucelus of vagus nerve(DMV) innervating. rat colon.***

1. DMV after PRV-BAblu injection into colon.
2. DMV after PRV-D injection into colon
3. PVN after PRV-D injection into colon
4. DMV coinfected by PRV-D followed PRV-Bablu injection into colon.
5. PVN coinfected by PRV-D followed PRV-Bablu injection into colon
6. DMV subjected to dual injection of the viral mutants into colon.

III : Third ventricle, → : PRV-D+PRV-Bablu, : PRV-Bablu, : PRV-D

BaBlu, functions to suppress replication of other strains of PRV that enter the cell subsequent to replication of PRV-D. The increased rate of replication and invasiveness of PRV-D relative to PRV-BaBlu provides a temporal advantage for PRV-D that is integral to the expression of this effect.

The virally encoded protein(s) present in PRV-D that suppress the replication of PRV-BaBlu remain to be identified. As noted earlier, the genetic manipulations used to produce PRV-D restored the gI gene to the Us region of the genome and it is possible that expression of this glycoprotein is instrumental in the suppression of subsequent PRV-BaBlu replication. However, rescue of the Us region of PRV during the course of PRV-D construction also restored two other gens, Us9 and 28k, that are lacking from both the parental virus and PRV-BaBlu. Brideau and colleagues (1998) have recently shown that Us9 is a phosphorylated tail anchored type II membrane protein that is highly conserved among the alpha herpesviruses. It localizes in membrane of intracellular secretory pathways, particularly the endoplasmic reticulum and Golgi complex, of PRV infected neurons early in the course of infection and is, therefore, in an ideal position to suppress the production and assembly of proteins essential for PRV-BaBlu replication. Little is known regarding the localization or function of the 28k gene product. Further work is necessary to determine if any of these genes contribution to the exclusion of PRV-BaBlu replication in cells previously infected with PRV-D.

Evidence also exits in our data in support of the interpretation that exclusion of one strain of PRV by another may be due to a blockade of fusion based entry of one strain and/or to exposure of permissive neuronal profiles to differing concentrations of virus, as previously demonstrated for HSV-1 by Johnson and Spear (1989). The presence of DMV neurons only infected with PRV-BaBlu after simultaneous injection PRV-BaBlu and PRV-D provided the clearest evidence in support of this possibility. In this experimental paradigm the majority of neurons infected by retrograde transport of PRV-D from the stomach far exceeded those that were co-infected or were only replication PRV-BaBlu. As noted above, we interpret the dominance of PRV-D infection to a block in PRV-BaBlu replication that occur subsequent to invasion of the two strains. However, the presence of DMV neurons that are only replicating PRV-BaBlu in this paradigm suggests that PRV-D either failed to invade these neurons of did no in such low concentration that a productive replication was not elicited. Either possibility is supported by the data and prior literature. For example, it seems quite probable that the diffusely ramifying axonal processes of DMV neurons will be exposed to variable concentrations of the two viruses such that some axons will be differentially exposed to high concentrations of only one virus. Under these circumstances. viral envelope proteins incorporated into the membranes of permissive processes could preclude or reduce the attachment and entry of other virions in manner analogous to that demonstrated in the in vivo studies involving HSV-1 and gD expressing cell lines(Campadelli-Fiume et al, 1988, 1990; Johnson & Spear, 1989). Given the recent finding that low concentrations can delay or eliminate the replication of PRV in a permissive neuron after intracerebral injection (Card et al, 1994) the differential invasion of higher concentrations of one strain of PRV could account for individual infection of neurons in dual infection paradigms. This would explain the persistence of cells differentially infected with PRV-BaBlu under circumstances in which PRV-D infection predominates.

These findings also have important implications for the design and interpretation of investigations involving the use of multiple strains of virus to define neural circuits. This approach has recently been employed using strains of HSV and PRV engineered to express unique gene products and is dependent upon the ability of two strains of virus to replicate in the same neuron. In studies of the organization of neuronal circuits that medulate the activity of the viscera and

homeostatic function, Jansen and colleagues (1995) and Levatte et al. (1998) have shown that retrograde transynaptic passage of recombinant strains of PRV and HSV-1 can be used to identify neurons in the central nervous system that synapse upon separate populations of spinal cord neurons that project to different peripheral targets. This is powerful exploitation of the neurotropism and invasiveness of alpha herpes viruses to define principles of synaptic organization that cannot be defined with other techniques. The present data set underscore the usefulness of this experimental approach but also reveal an important aspect of the alpha herpesvirus life cycle that should be considered in both the design and interpretation of data derived from such studies. In particular, the ability of one virus to exclude the replication of another would create false negatives that would underestimate the number of neurons that contribute to a polysynaptic circuit. Thus, caution should be exercised in interpreting negative findings involving this experimental approach. The data also suggest that the best results would be achieved through the use of isogenic strains of virus that are equivalent in virulence and rates of invasiveness.

V. Conclusion

Genetically engineered development of pseudorabies viruse(PRV) as vectors for delivery of foreign genes to postmitotic neurons in the dorsal motor nucleus of vagus nerve(DMV) of central nervous system(CNS) has also made it possible to use more than one strain of virus in complex tracing paradigms after injection of these viruses into rat proximal and distal colon. In the present investigation we used two genetically engineered strains of PRV in single and dual injection paradigms using two in vivo rodent models. Both viruses are isogenic with the Bartha strain of PRV(PRV-Bartha), but have been constructed to produce unique gene products that can be detected immunohistochemically. In one strain, PRV-BaBlu, the bacterial lacZ gene was placed in the gG locus of the unique short (Us) region of the viral genome; β-galactosidase expression provides the unique marker of neurons infected with this strain. The other strain, PRV-D, was constructed in two steps. First, the deletion in the unique short region of PRV-Bartha was repaired with wild type DNA from PRV-Be(PRV Becker strain). The results were as followings.

First, the progression of infection resulting from individual inoculation with PRV-D is significantly faster that resulting from individual inoculation with the parental strain or PRV-BaBlu in the same circuitry.

Second, virulence of the engineered mutants differs substantially from one another and the parental virus. PRV-D is more virulent than either PRV-Bartha or PRV-BaBlu, and PRV-BaBlu is less virulent than PRV-Bartha. Third, neurons in DMV innervating rat colon are permissive to coinfection by both mutants when the viruses invade simultaneously.

< References >

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