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Application of in situ viral nucleic acid hybridization to the study of Aujeszky's disease (pseudorabies) in calves

Harold Antonio McAllister

Iowa State University

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APPLICATION OF IN SITU VIRAL NUCLEIC ACID HYBRIDIZATION TO THE
STUDY OF AUJESZKY'S DISEASE (PSEUDORABIES) IN CALVES

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Application of in situ viral nucleic acid hybridization to the study of Aujeszky's disease (pseudorabies) in calves

by

Harold Antonio McAllister

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

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GENERAL INTRODUCTION

Pseudorabies is a viral infection that affects the central nervous system. It is primarily associated with swine because they can either develop inapparent infections or remain latently infected and shed virus after clinical recovery, while in other domestic species the disease is usually fatal (4). Species other than swine therefore do not play significant roles in the spread of the disease, and its presence is dependent on the presence of swine (4). Since pork production is a major industry in Iowa and swine are the major reservoir of this virus, it is not uncommon to encounter cases of pseudorabies in the state's cattle.

This dissertation, which deals with the problem of bovine pseudorabies, is presented in the alternate format. It consists of three manuscripts that meet style requirements of the Journal of Histochemistry and Cytochemistry, a refereed journal. The second manuscript has already been published (7), and the third one has been submitted for publication. Although the three manuscripts are interrelated, each one is self-contained and independent of the others.

The first manuscript represents a critical rather than a historical review of much of the literature available on bovine pseudorabies. It focuses on the problem from the perspective of pathogenetic mechanisms, highlights opportunities for further research, raises questions about accepted views, and suggests that recently developed techniques may prove useful in gaining new insights about this particular infectious process.

One of the newest techniques for elucidating viral pathogenesis is in situ nucleic acid hybridization as outlined by Brahic and
Haase (1). The second manuscript in the dissertation deals with the student's efforts to adapt the technique for its use in cattle. This work was actually carried out in murine tissues. A biotechnologic tool known as a nucleic acid probe is used and it was essential to establish that the available probes could detect the nucleic acids of pseudorabies virus effectively and specifically. In addition, there was a pressing need to find alternative methods of tissue fixation.

Detachment of tissues from histopathologic slides during in situ hybridization, particularly those fixed in either formalin or periodate-lysine-paraformaldehyde (PLP), was at first a major obstacle. Moreover, PLP, commonly used for in situ hybridization, was originally designed for small tissue pieces as used in immunoelectron microscopy (8). A fixative capable of penetrating the large bulk represented by half a bovine brain plus assorted and equally bulky tissues was essential.

Fixation with formaldehyde/paraformaldehyde involves an unavoidable equilibrium of three chemicals that can be expressed as follows:

\[
\begin{align*}
\text{methylene glycol} & \leftrightarrow \text{carbonyl formaldehyde} \leftrightarrow \text{paraformaldehyde} \\
\text{H}_2\text{O} & \quad \text{OH}^- \\
\text{H}^+ &
\end{align*}
\]

Methylene glycol penetrates tissues rapidly but it has no significant fixing action (3). Carbonyl formaldehyde is a good fixative, but acidification of the medium causes it to polymerize back to paraformaldehyde, an insoluble solid (9). Oxidation of carbonyl formaldehyde by air and tissue components to formic acid, acidification of the solution by \( \text{CO}_2 \) dissolved from air, and modification of the
alkalinity of the fixative solution in the microenvironment of the tissues can all shift the equilibrium away from methylene glycol. Conditions ideal for fixation with this system may thus hamper its penetrability (2). Formaldehyde is sold mixed with a stabilizer to prevent polymerization (9), but there is no such stabilizer in PLP. Moreover, the amount of paraformaldehyde in PLP is only 2% (8).

Alternative methods of fixation, such as fixation by coagulative fixatives (6), were successfully tried and Carnoy's fluid seemed promising. Unfortunately, classical Carnoy's fluid hydrolyzes nucleic acids (6) such as those that the probes seek to detect and it also contains chloroform, which must be removed after fixation (5). The formulation of the fluid was therefore modified and this modified Carnoy's fluid (MOCA) proved ideally suited for the intended work after procedures were developed to minimize its tendency to hydrolyze nucleic acids.

The objective of the second manuscript of the dissertation is to offer a detailed account of the various fixatives tested for degree of usefulness and compatibility with in situ hybridization. The third manuscript documents the reproducibility of results obtained when in situ hybridization was applied to a wide range of MOCA-fixed, pseudorabies-infected bovine tissues. Their reliability was measured by comparison with concurrent quantitative virus isolation. In addition, the third manuscript offers the result of a limited retroactive study carried out with 7 year old formalin-fixed infected neural bovine tissues.

The Iowa State University Committee on the Use of Animal Subjects
in Research reviewed and approved funding for this project and concluded that the rights and welfare of the animal subjects were adequately protected.

The Ph.D. candidate, Harold Antonio McAllister, is the sole author of the first manuscript and the principal investigator for each of the studies reported in the subsequent two manuscripts. Acknowledgments for technical assistance and for contributions by co-workers follow each paper. Additional acknowledgments are presented at the end of this dissertation.
LITERATURE CITED


SECTION I. PSEUDORABIES IN CATTLE: A REVIEW
Pseudorabies in cattle: a review

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ABSTRACT

Pseudorabies, also known as Aujeszky's disease, is a viral infection that affects the central nervous system of most mammals. This review of the literature offers a general picture of the clinical condition as seen in cattle, deals with the discovery of its association with swine, and looks critically at the research published on the pathogenesis of bovine pseudorabies. The viral portals of entry, the localization and replication of the virus, the viral effects on its host, and the host's response to the infection are discussed individually.
INTRODUCTION

In 1902, the Hungarian scientist Aladar Aujeszky described a disease similar to but different from rabies in an ox, a dog, and a cat (5). He recorded the clinical signs, proposed that the causative agent gained access to the host through damaged skin or oral mucosa, reproduced the condition in rabbits given emulsified brain tissues from infected animals, and noted lack of contagiousness among the laboratory animals. One of the early names for this condition was infectious bulbar paralysis (6, 44) but today it is best known as either Aujeszky's disease (AD) or pseudorabies (PR).

Research done in the U.S. by Richard Shope in the 1930s (53, 54) strongly supported the concept of swine as the natural host species for the agent of AD, as well as the propriety of classifying it as a filterable entity or virus. In addition, using a virus strain supplied to him by Dr. Aujeszky, Shope established the identity between AD and "mad itch", a disease of cattle recognized in the U.S. since the 1890s.

Pseudorabies virus (PRV) belongs in the family Herpesviridae, subfamily alphaherpesvirinae (48, 49). Due to its intimate involvement with pigs, it is specifically classified as Suid herpesvirus 1 (48, 49). Alpha-herpesviruses can have a wide host range. Typically, they have a short reproductive cycle, spread rapidly in tissue cultures (destroying the susceptible cells), and frequently establish latent infections in ganglia. The PRV virion is an enveloped particle with a diameter of 120 to 200 nm, a double stranded linear DNA genome, icosahedral capsid
symmetry, and over 20 associated structural polypeptides (16, 48, 49).

Taxonomically, PRV is similar to a bovine-adapted alphaherpesvirus, Bovid herpesvirus 1, which is the cause of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis in cattle (48, 49). Antigenic kinship (1, 2, 9, 10, 16, 34, 55, 65, 66) and genomic similarities (15) between infectious bovine rhinotracheitis virus (IBRV) and PRV have been documented.

The recognition of sporadic field cases as well as epizootics of pseudorabies in cattle has led to numerous reports from continental Europe, the British Isles, and both North and South America (1, 4, 6, 7, 8, 11, 12, 13, 14, 17, 18, 19, 20, 21, 22, 23, 25, 27, 29, 30, 31, 33, 34, 35, 36, 38, 39, 40, 44, 46, 50, 51, 52, 53, 57, 58, 59).
CLINICAL PICTURE OF BOVINE PSEUDORABIES

Cattle infected with PRV are clinically normal during an incubation period of variable length that tends to average 5 to 7 days (3, 4, 9, 10, 13, 17, 20, 25, 26, 32, 33, 40, 42, 43, 53, 61, 62, 63, 66). Attempts to isolate virus at sites distal to the inoculum site during that period have been unsuccessful (9, 32, 42). The disease is fulminating. Animals, especially calves, die within 6 to 36 hours after the first clinical signs appear (4, 8, 9, 10, 11, 17, 20, 25, 26, 29, 31, 32, 33, 34, 35, 39, 40, 43, 44, 51, 53, 54, 61, 63, 66). Sometimes death is sudden and no significant clinical signs are observed; the animals are just found dead (25, 43, 60, 63). Most workers consider the disease fatal (2, 4, 8, 9, 10, 14, 33, 34, 36, 38, 57, 59). Prolonged illness (22, 31, 43, 53) has rarely been recorded. Reports of nearly inapparent AD, of recovery from natural PRV infection, or of survival after challenge in the absence of preexisting immunity in cattle are few (19, 30, 34, 60), and they have been questioned. Nonetheless, attempts at immunizing cattle with both attenuated and inactivated vaccines are increasing (3, 9, 10, 24, 37, 38, 45, 47, 55, 56, 60, 64, 65, 66) and vaccination trials have led to reports of safe challenge with virulent PRV after immunization against AD (45, 56, 66), so survival is possible. Unfortunately, even attenuated PRV has been reported to cause disease in cattle (50, 66), and immunization cannot guarantee complete protection against virulent virus (3, 9, 10, 60, 66).

Typical AD clinical signs in cattle include anorexia, excessive
salivation, bloating, restlessness, hyperesthesia, localized pruritus, and, in some cases, fever (4, 7, 8, 19, 20, 25, 27, 31, 32, 33, 35, 39, 40, 43, 44, 51, 53, 57, 59, 60, 61, 66). The development of intense itching with a frenzy of self-mutilation, which led to the name of "mad itch", does not always occur (4, 7, 11, 19, 25, 26, 43, 53, 60, 61, 62, 66). When itching does occur, it tends to be either anterior or posterior on the animal's body; its location is influenced by the viral mode of entry and, to a certain degree, by the specific point of viral access to the host (8, 11, 12, 13, 17, 20, 32, 40, 53).

Gross necropsy lesions other than those due to self-mutilation are either absent or few, minor, and nonspecific, such as occasional hemorrhages or edema in various organs (7, 20, 25, 26, 29, 30, 31, 35, 39, 40, 44, 51, 53, 61, 62, 63). Histopathologic lesions specifically attributable to AD are limited to neural tissues, particularly ganglia, the spinal cord, and the brain (3, 7, 8, 19, 20, 21, 25, 26, 28, 30, 42, 44, 51, 61, 62, 63). Occasionally, microscopic neural lesions cannot be found (7, 35, 52). Since the distribution of lesions may be focal or multifocal, being restricted, for example, to a segment of the spinal cord, their apparent absence could stem from the impracticability of taking enough samples to examine in minute detail an entire bovine nervous system. The lesion most universally reported is that of subacute lymphocytic inflammation of neural tissues, with edema and perivascular cuffing. Other histopathologic lesions noted include neuronal degeneration, satellitosis, eosinophilic nuclear inclusions (mostly in neurons), gliosis (especially as glial nodules), microhemorrhages, plus microfoci of
necrosis infiltrated by lymphocytes and occasional neutrophils. The most complete descriptions of microscopic lesions in bovine AD published so far are those of Dow and McFerran (25, 26).
NATURAL RESERVOIRS OF PSEUDORABIES VIRUS

In 1935, armed with clues from Hungarian and Brazilian observers supplemented by careful research and thought of his own, Shope (54) postulated that swine were the source or reservoir of PRV for cattle. He realized that in swine the manifestations of PRV infection vary, sometimes being mild or even inapparent, and that in this sense swine were quite different from other domestic and laboratory animals susceptible to fatal AD. He confirmed that in swine AD is contagious and that infected pigs carried readily transmissible virus in their nasal secretions; he also downplayed the then widely held notion of rats as crucial vectors in the epidemiology of the disease. Doubters of Shope's views on pigs as a PRV reservoir arose periodically (8, 41, 57), apparently as a result of mistakenly equating absence of disease in swine to absence of infection. In 1947, Lamont (41) in Ireland championed a cycle involving rats and went so far as to state that "the fact, too, that pigs develop a potent antiserum on infection hardly suggests this animal as a reservoir of the virus." Fortunately, ample evidence in support of the role of pigs as a reservoir of PRV has accumulated since Shope's day (4, 7, 12, 14, 18, 27, 33, 35, 36, 38, 44, 51, 52, 58, 60), and his view is now universal.

In 1964, McFerran and Dow (43) noted that they could not detect PRV in the nasal secretions of infected cattle. Their finding generated the view that cattle are of no epidemiological importance in bovine pseudorabies and that they cannot act as a source of infection (63). This view
is at odds with recent reports of the presence of PRV in the nasal secretions of a number of infected cattle (11, 13, 20, 32, 63). Notably, Wittman et al. (61, 63) have found evidence of substantial amounts of virus with apparent multiplication in the nasal cavity of intranasally infected cattle that are over a year old. The titers are lower than those seen in the nasal cavity of pigs (20, 61, 63), but they are high enough to preclude continuing to ignore the possibility of cow to cow transmission. The titers reported range up to $10^{3.5}$ median tissue culture infective doses (TCID$_{50}$) per nasal swab. For comparative purposes, we should note that the median lethal dose (LD$_{50}$) of certain PRV strains for intranasally infected cattle has been estimated at $10^{4.6}$ TCID$_{50}$ by Biront et al. (9, 10) and at $10^{3.2}$ plaque forming units (PFU) by Van Oirschot et al. (60).
PATHOGENESIS OF BOVINE PSEUDORABIES

The Portals of Entry

In intact mammals, the possible portals of entry for any infectious agent are the skin, the digestive tract, the respiratory tract, the conjunctiva, and the orifices of the ears and the genitourinary tract. Hopp et al. (32) found that undamaged skin, eyelids, or forestomach mucosa successfully protected cattle from PRV doses over fifty million times greater than those required to establish infection by subcutaneous inoculation.

After Shope (54) showed that the pig's nose is well-suited for both the entrance and exit of PRV, he rubbed the noses of infected swine against the scarified skin of experimental rabbits and successfully transferred AD to them. Shope's following words about cattle and swine (54) deserve to be quoted:

To one familiar with the behavior of swine when they are with cattle, it seems likely that a virus present in the nose of a hog, or more especially on the nose, could be transferred to the skin of a cow. Cattle lying about a barnlot in which hogs are also kept come frequently in contact with the pigs' noses. Swine under such conditions can be observed to approach a cow and probe it in the flank or side with their noses. If the first punch fails to get the cow up, a second and more vigorous probe is given. This process is continued more persistently, and not infrequently, as a last resort, they will bite the cow, often through the areas of skin probed with their noses.

Although Lamont (41) underrated the role of pigs, he proposed that natural PRV infection occurs through minute abrasions of the skin and added that thereafter infection appears to travel through nerves to the corresponding ganglia and sectors of the spinal cord. This notion was strongly supported by Dow and McFerran (25, 26) and McFerran and Dow (43)
in the 1960s and by McCracken et al. (42) in 1973. The picture of the classical "mad itch" of cattle thus became clearer. However, by the 1970s the barnyards of Shope's day with their mixtures of pigs and cows were largely gone, and intimate contact between pigs and cattle was the exception, not the rule. Yet, bovine pseudorabies persisted.

Alternative modes of infection had to be considered. Although Dow and McFerran infected calves by a number of different routes, they appear to have considered them all minor variations of a single theme and did not elaborate on their relationship to field conditions. Bitsch (11, 12, 13) sought to bridge the gap from the laboratory to the field in 1975 by studying 29 outbreaks of AD in cattle. He classified them into those with anterior pruritus and those with pruritus in the posterior part of the body, and noted that the animals with anterior pruritus were more numerous. Bitsch (11) proposed that the mucous membranes might be sites of entry and of multiplication of PRV. In his analysis of the outbreaks (12, 13), he concluded that airborne virus could lead to respiratory infections with anterior pruritus, that genital infections with posterior pruritus could be unintentionally set up by human vectors, that a few infections may have been of alimentary origin, and that none of the cases suggested cutaneous infection.

In 1985, Hopp et al. (32) confirmed that the spread of PRV in the bovine body and the ensuing clinical picture are determined by the viral portal of entry. Intranasal or intrabronchial instillation, as well as aerosol inhalation, led to virus dispersion in the neural tissues of the head and in the cervical portion of the spinal cord. Introduction of
virus to the undamaged mucosa of the rectum or vagina, lumbar subcutaneous injection of the virus, or oral administration led to caudal virus dispersion; in these cases PRV could not be shown to have reached either the brain or spinal cord levels anterior to the first thoracic vertebra. Cranial virus dispersion resulted in a short illness with head itching, restlessness, neck spasms, local paralysis of the head, and rumen tympany (bloat). Caudal dispersion resulted in a longer illness with mild posterior pruritus, colic, and abdominal muscle spasms. The results reported by Hopp et al. (32) after oral administration of PRV do not, however, match those reported by McFerran and Dow (43), who did not notice an ensuing caudal dispersion.

Localization and Replication of the Virus

The work of Dow and McFerran in 1962 (25) and of McFerran and Dow in 1964 (43) suggested centripetal spread of PRV via nerves to their ganglia and to the spinal cord or brain depending on the inoculation site. Moreover, they failed to recover virus from liver, spleen, kidney, lungs, heart, various lymph nodes, adrenal glands, pancreas, blood, throat swabs, nasal swabs, rectal swabs, and urine from animals inoculated subcutaneously, intradermally, intranasally, or orally. They therefore became convinced that in cattle PRV is strictly neurotropic (26, 42), a view that was openly questioned by Bitsch (11). Bitsch also noted that the site of pruritus needs not be identical to the site of viral entry.

Review of the literature on bovine pseudorabies reveals that in addition to virus in the nasal cavity, there are reports of PRV isolation
from the oral and pharyngeal mucosa (11), from the tonsil (20), from the thymus (63), from the esophageal and tracheal mucosa (32), from lymph nodes (26, 63), from the adrenal glands (26), from parenchymatous organs such as spleen and liver (23, 53, 57), from blood (22, 23), and, particularly, from lungs (7, 13, 32, 40, 53). Some of these reports, all of which involve either natural infection or inoculation by routes other than the intravenous, may be more reliable than others. Most are subject to the criticism that the structures involved have nerves of their own, but the reports should not be casually dismissed. It may prove fruitful to look for virus again where it is not expected, using today's more sensitive methodologies. Interestingly, Dow and McFerran's (26) virus isolation results after intravenous inoculation are not strikingly different from those reported by Hopp et al. (32) with infection by aerosol.

The possibility of a single cycle of replication on a mucosal surface, followed by a short, disastrous, blood-borne shower of virus upon the replication-permissive tissues of the central nervous system ought to be carefully evaluated.

Researchers studying the effectiveness of vaccination against AD in cattle (60) have produced some evidence that the length of the incubation period after intranasal instillation of virus is dose-dependent. Among 19 control animals, the mean length of the incubation period was increasingly shorter as the challenge dose increased. For four animals given 6000 LD$_{50}$ of PRV, the mean incubation period was only $99 \pm 6$ hours. Other researchers (61, 63) have found that the duration of survival after nasal inoculation may also be inversely related to dosage. A calf given
a large dose of PRV may perish in as few as 3 days. Moreover, the distribution of the virus appears to depend on the time of death (61, 63), and in calves dying soon after infection it can be absent from the brain and present only in nasal mucosa and thymus. These findings suggest that primary viral replication can occur outside the central nervous system, particularly in the nasopharyngeal mucosa, and that for some reason this event is lethal.

Permissive tissues, that is, bovine tissues that allow PRV to replicate, might be limited to certain ectodermal (brain, spinal cord, peripheral ganglia, nerves, nasal and olfactory epithelium) and perhaps some endodermal derivatives (pharyngeal and respiratory epithelium, thymus). There could be variability in permissiveness, with the determinant being, for example, the relative abundance of a host-derived membrane fusion factor. In non-neural bovine tissues, this factor could be partially inducible, as opposed to being readily available in the tissues of some other domestic animal species. Tissues well-endowed with cells rich in this hypothetical factor would allow the virus to infect them quickly and to initiate replication. In this model, the higher the infective dose, the higher the likelihood that the virus will infect a critical mass of susceptible cells promptly, even in tissues not as well-endowed with those cells as the brain or spinal cord.

The permissiveness of the central nervous system of cattle to PRV is well-established. Isolations of the virus from brain (7, 8, 9, 10, 11, 19, 20, 23, 26, 30, 32, 35, 39, 40, 42, 43, 51, 53, 59, 61, 63) and from spinal cord (7, 8, 11, 20, 21, 29, 32, 39, 42, 44, 61, 63, 66) are
regularly reported. As noted in the preceding discussion, however, the
dispersion of the virus is both route and dose-dependent, so its distribu­
tion in the body is not homogeneous. Negative viral isolations from
the brain (7, 11, 17, 29, 32, 35, 42, 44, 61, 63) and from spinal cord
(8, 20, 26, 32) are therefore not surprising.

Viral Effects on its Host

The presence of PRV in nervous tissues can lead to considerable
damage. This is reflected by the type of histopathologic lesions alluded
to in the discussion of the clinical picture of bovine pseudorabies.
Viral replication leading to cell death or necrosis can be expected to be
involved in the production of damage, but other mechanisms may also be at
work.

Large numbers of lymphoid cells infiltrating infected nervous tissue
suggest that cellular immunity has been induced. Whether they are
responding to self-antigens released from immunologically privileged
sites by the necrotizing process, responding to the virus and the virus-
harboring cells, or responding to both has not been critically examined.

An implicit assumption of absolute correspondence between histo-
pathologic lesions and viral presence seems to underlie the existing
literature, and it may be wrong. Virus-induced damage may be indirectly
amplified by immune processes. Whether this may happen or whether minute
amounts of virus below the detection threshold of our most sensitive
techniques are also responsible for damage should be investigated.

Tissue destruction within the central nervous system can lead to
disruption of signal matrices by blocking ascending and descending pathways. Severe consequences should be expected. The effects of viral proliferation in individually distinct regions of the bovine brain and spinal cord have not been examined, however. The specific effects of viral infection and its associated inflammation in individual susceptible ganglia have not been examined either.

The nature of the lethal event or events in bovine pseudorabies has not been investigated. Precisely what does the virus do that can be lethal to a calf within 3 days postinfection? Why does central nervous system damage that is similar to but less extensive than that seen in the encephalitides of other mammalian species kill a steer within hours? How can so much damage develop so quickly? How can damage that is restricted to the lumbar spinal cord be fatal? Even more interesting is to consider what is the lethal event in PRV-infected cattle that die with no significant lesions either in the brain or the spinal cord. Is viral replication at an undetected site releasing a biochemical factor that by itself blocks the functioning of key portions of the bovine nervous system? Alternatively, has a severe but undetected ganglionic infection destroyed a neural signal relay station essential for respiration or rumination or both?

Host Response to Pseudorabies Infection

Since the horizontal transmission of PRV in cattle is not believed to be a major epidemiological mechanism (20), and since cattle are not normally exposed to this lethal virus, the survivors of an epizootic
(presumably still unexposed) do not normally seroconvert (9, 14, 20, 36, 38, 60), but there may be exceptions (30). Cattle appear unable to mount a significant protective immune response to PRV in the face of infection by virulent strains. However, preexisting immunity as a result of vaccination with an inactivated vaccine can effectively protect cattle from low intranasal doses of virulent PRV (60). In addition, vaccination significantly lengthens the incubation period that precedes the first clinical signs after intranasal administration of high doses of the virulent virus (60). The bovine immune response to PRV is therefore not totally ineffective; it appears to be primarily directed toward the formation of humoral neutralizing antiviral antibodies, but this appearance may be due to bias in our testing procedures. Reports of cattle successfully protected by modified live PRV vaccines exist (37, 38, 45, 56, 64), and a live virus presumably would stimulate both humoral and cellular immunity.

Considering the taxonomic, genetic, and antigenic similarities between PRV and IBRV, it is not surprising that the key to bovine survival after challenge by PRV has been reported to lie in immunity to IBRV (1, 2, 9, 10, 34, 55, 60, 65, 66). Cattle primed to IBRV can have PRV neutralizing antibodies even if they have never had contact with the latter virus (1). Joubert et al. (34) suspected that concurrent latent infection with IBRV during a bovine enzootic of AD had made possible the existence of several mild cases of PRV infection in cattle. Van Oirschot et al. (60) found that steers with preexisting PRV neutralizing titers, presumably due to IBRV infection, reacted serologically after primary
vaccination with PRV as if they were undergoing a secondary response. They also noted that steers initially seronegative to PRV but seropositive to IBRV produced higher postvaccinal anti-PRV titers than cattle initially seronegative to either virus. In addition, in their study, 4 cattle that had anti-IBRV titers and were vaccinated once with PRV resisted challenge with a low dose of virulent PRV, while 5 IBRV seronegative steers given the same treatment developed AD and died.

Significant serological cross reactivity between IBRV and PRV was first documented by Aguilar-Setién et al. (1, 2) in 1979. They noted that large numbers of bovine sera devoid of neutralizing activity toward IBRV also lacked neutralizing antibodies against PRV. In contrast, 30 out of 90 sera from cattle that were either naturally infected with IBRV or vaccinated against it had concurrent neutralizing activity against PRV. When PRV antigens were injected into IBRV primed cattle, the serologic neutralizing titers against both viruses rose (1); however, such antigens did not seem to enhance anti-IBRV titers in unprimed cattle. A unidirectional cross reactivity phenomenon of this sort was also reported by Straub et al. (55) in 1983, but they did detect slight increases in anti-IBRV titers after vaccinating cattle with an inactivated PRV vaccine. Žuffa et al. (65, 66), on the other hand, found low but measurable heterologous neutralizing activity after immunizing cattle with either inactivated PRV or IBRV; they also demonstrated that calves with high titers of antibodies to IBRV could survive primary intranasal challenge with a substantial dose of an attenuated PRV strain.

In 1986, Bush and Pritchett (16) found that PRV and IBRV bear a
number of cross-reactive proteins and that there is at least one common antigenic determinant which elicits neutralizing antibodies. Apparently, IBRV is far more efficient at triggering production of those antibodies than PRV. Bush and Pritchett (16) reported that IBRV hyperimmune serum reacted strongly with 9 PRV proteins, whereas PRV hyperimmune serum reacted strongly with 3 and weakly with 2 IBRV proteins.
CONCLUSION

The clinical picture of bovine pseudorabies and its pathogenesis have been reviewed in the hope of achieving a deeper or more refined understanding of the process. Opportunities for additional research were highlighted, as well as questions stimulated by accepted views. It should be clear to the reader that we are far from having a complete and detailed understanding of the interaction between the virus and its host. The tools and techniques of biotechnology should be applied in order to further that understanding. We must remain alert to the possibility that the constellation of viral and host activities may offer a chance for veterinary therapeutical intervention. We should investigate why PRV rarely produces disease in horses, why the tailless apes do not seem to be susceptible to it, and why it is so lethal in species such as cattle. The lessons we may learn from a fatal alphaherpesvirus infection in the bovine might even help us to deal with all of the alphaherpesviruses, which include the *Herpes simplex* viruses of humans.
LITERATURE CITED


2. Aguilar-Setién A, Vandeputte J, Pastoret PP, Michaux C, Pensàert MB, Schoenaers F: Présence concomittante, chez les bovins et les porcs, d'anticorps neutralisant le virus de la rhinotraceite infectieuse bovine (Bovid Herpesvirus 1, BHV 1) et celui de la maladie d'Aujeszky (Sus Herpesvirus 1, SHV 1), après contact avec le virus homologue. Ann Méd Vét 123:275, 1979


44. Morrill CC, Graham R: An outbreak of bovine pseudorabies or "mad itch". Am J Vet Res 2:35, 1941


65. Žuffa A, Zajac J, Žuffa T: A study in calves, swine and rabbits of the immunological relationships between infectious bovine rhinotracheitis virus and Aujeszky's disease virus. Zentralbl Veterinarmed (B) 30:211, 1983

SECTION II. COMPARATIVE USEFULNESS OF TISSUE FIXATIVES FOR
IN SITU VIRAL NUCLEIC ACID HYBRIDIZATION
Comparative usefulness of tissue fixatives for in situ viral nucleic acid hybridization

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ABSTRACT

Traditionally, tissues for in situ hybridization of viral nucleic acid have been small pieces obtained from laboratory rodents, and fixatives that are designed for electron microscopy, such as periodate-lysine-paraformaldehyde (PLP), can handle them adequately. However, these fixatives have limited penetrating ability and may produce no appreciable hardening, so alternative fixation methods were evaluated. The intention was to determine whether fixatives adequate for bulky tissues such as whole or halved pig and cow brains would also be compatible with in situ hybridization. Various fixatives were evaluated using a system of intracranial inoculation of BALB/c mice with pseudorabies virus (PRV) followed by in situ hybridization of brain tissue sections with a $^{35}$S-labeled PRV DNA probe. Loss of tissue sections was a major problem, particularly with PLP and formalin, but positive results were obtained with five fixatives tested. Cellular morphology was especially good with PLP and with a modification of Carnoy's fluid, MOCA fixative. An incidental but important observation was that formalin is compatible with in situ hybridization. Retroactive studies of viral diseases using routinely processed blocks of tissue (formalin-fixed, paraffin-embedded) are therefore conceivable.

KEY WORDS: Fixatives for in situ hybridization compared; In situ hybridization with $^{35}$S-labeled pseudorabies DNA probe; In situ hybridization of formalin-fixed tissue; Mice, inbred BALB/c, infected with
pseudorabies virus; Modified Carnoy's (MOCA) fixative; Periodate-lysine-paraformaldehyde (PLP) fixative; Pseudorabies virus $^{35}$S-labeled DNA probe for in situ hybridization.
INTRODUCTION

In situ hybridization of viral nucleic acids is increasingly important in the study of disease because it permits detection of minute amounts of virus in tissue sections (1). Virus particles are localized by autoradiography, and the number of silver grains that develop at the site is roughly proportional to the degree of hybridization (1, 8). Few fixatives have been used in conjunction with this technique. They include ethanol-acetic acid (1, 8, 9), glutaraldehyde (2, 3), and periodate-lysine-paraformaldehyde (PLP) (10). The latter was originally developed for immunoelectron microscopy (7). Fixatives intended for use in electron microscopy, such as glutaraldehyde, have limited penetrative ability. They are adequate for small pieces of tissue from laboratory rodents but are inappropriate for bulky material from larger experimental animals, such as brains from older calves.

We examined a variety of fixation methods in order to identify fixatives useful for in situ hybridization of herpesviruses in bovine and porcine tissue. We were aware that different fixatives are ideal for different body tissues (4, 5). Formalin was of some interest to us because of its widespread popularity in routine fixation procedures. Moreover, if it were found to be compatible with in situ hybridization, the possibility of retrospective studies of innumerable diseases could be considered.

In situ hybridization seeks to detect virus while preserving tissue integrity to an extent greater than is possible on sections cut from
frozen blocks. Specific cells involved in the infectious process are, therefore, more easily identified and recorded. Since small amounts of virus are detectable within wide expanses of tissue, anatomic structures that should be further examined by electron microscopy are pinpointed.

Ideal tissue fixation often requires mixtures of protein coagulants such as ethanol and of noncoagulative aldehydes (4, 5). Mixtures designed with components that counter each other's weaknesses, that is, balanced fixatives such as Bouin's (4), are not as popular as formalin but are nonetheless useful. The purpose of this report is to compare the usefulness of several balanced fixatives and of formalin to that of PLP for in situ hybridization. The fixatives were evaluated using a mouse brain infection system with pseudorabies virus (PRV).
MATERIALS AND METHODS

Infection of Animals

The Becker strain of PRV was obtained from Dr. K. Platt at Iowa State University and viral stocks were stored frozen at -70°C. Fifteen BALB/c female mice 4-6 weeks old weighing from 11 to 18 g were anesthetized and infections were initiated by the intracranial midline injection of $5 \times 10^5$ TCID$_{50}$ of PRV in 0.05 ml of 1 x minimal essential medium per animal. An additional 15 animals of the same type were maintained as uninfected controls; no intracranial injections of saline were given to them.

Fixatives

McLean and Nakane's method (7) was used to prepare PLP buffered to pH 7.4. After preliminary experiments with various standard fixatives, three acidic and one other buffered fixative were prepared as in Table 1.

Fixation of Tissues

Brains were dissected out whole from five infected mice that died spontaneously between 21 and 23 hours postinoculation (PI) and from all surviving infected and control mice, which were killed by cervical dislocation starting at 22 hours PI. Three infected and three uninfected brains were immersed separately in each one of the five fixatives, with each group of three brains in its own 50 ml of fixative.

Fixation in MOCA and BAFI lasted 5 hours, whereas FOAL, formalin,
Table 1. Compositions of tested fixatives

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Modified Carnoy's (MOCA)</th>
<th>Formol alcohol (FOAL)</th>
<th>Balanced fixative (BAFI)</th>
<th>Neutral buffered formalin-saline (FORM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute ethanol, %</td>
<td>73.5</td>
<td>64.0</td>
<td>68.0</td>
<td>0</td>
</tr>
<tr>
<td>Type I water, a %</td>
<td>0</td>
<td>27.0</td>
<td>20.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Glacial acetic acid, %</td>
<td>24.5</td>
<td>4.5</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>37% Formaldehyde, %</td>
<td>2.0</td>
<td>4.5</td>
<td>7.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$.$H_2$O, g/liter</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>4.0</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$, anhydrous, g/liter</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>6.5</td>
</tr>
<tr>
<td>NaCl, g/liter</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>8.5</td>
</tr>
</tbody>
</table>

aNational Committee for Clinical Laboratory Standards (771 E. Lancaster Ave., Villanova, PA 19085), publication C-3.
and PLP fixation lasted 20 hours; the lengths of these fixation periods were selected on the basis of the size of the tissue pieces and the nature of the fixative employed. The tissues had to be stored for several hours or more before further processing, so additional manipulation was required to remove residual acetic acid and to prevent the excessive hardening and brittleness associated with storage at very high ethanol concentrations. Tissues fixed in MOCA went directly to 100% ethanol (EtOH) and then were partially rehydrated by successive 30-minute washes in graded alcohols (95, 85, and 80% EtOH). Tissues fixed in BAFI and FOAL went through 30-minute washes in 85 and 80% EtOH. Formalin- and PLP-fixed tissues were only washed once for 30 minutes in 70% EtOH. Final storage of all trimmed tissues in cassettes, prior to dehydration, clearing, paraffin infiltration, and embedding, was in clean 70% EtOH.

In Situ Hybridization

The in situ hybridization procedure of Brahic and Haase (1) entails the following steps: preparation of a radioactive probe, special chemical treatment of glass slides, generating tissue sections, pretreating them, nucleic acid denaturation, hybridization, autoradiography, and staining. Our method was adapted from theirs.

A PRV $^{35}$S-labeled DNA probe was prepared by means of a 25 µl nick translation reaction. The reaction mixture contained 1.0 µg viral DNA, 1.25 µg bovine serum albumin, 500 pg DNase, 5 units of DNA polymerase I, 4.5 µM deoxyadenosine 5'-($\alpha$-[$35$S]thio)-triphosphate, 2.2
mM dGTP, 2.2 mM dTTP, 2.2 mM dCTP, 5 mM Mg Cl₂, and 0.1 mM dithiothreitol in 50 mM Tris HCl, pH 7.8. After incubation at 15°C for 2 hours, the reaction was stopped by first adding 100 µl of a buffer containing 0.1% sodium dodecyl sulfate (SDS), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM Tris HCl, pH 7.4, and then adding 50 µg of salmon sperm DNA. The ³⁵S-labeled DNA was separated from unbound nucleotides by gel filtration on a G-50 Sephadex (Pharmacia, Piscataway, NJ) column followed by ethanol precipitation. The material was stored at -20°C until needed.

Microscope slides used in our experiments were treated with Denhardt solution and acetylated to decrease nonspecific adherence of probe DNA to glass (1, 8). Tissue blocks were infiltrated with Surgipath Embedding Medium EM-400 (Surgipath Medical Industries, Northbrook, IL) and cut at room temperature with a Reichart-Jung square-back microtome knife (Shandon Southern Instruments, Sewickley, PA). The standard-thickness sections (5 µm) were then collected on slides by flotation in water at 40°C containing 0.1% Elmer's white glue (Borden, Columbus, OH) to enhance adherence to the glass. The sections were deparaffinized in xylene and rehydrated just before pretreatment.

Pretreatment consists of steps that enhance diffusion of the probe into tissue by mildly modifying the permeability of cell surfaces (1); hybridization of the probe to viral nucleic acid within cells is thus maximized. In the usual procedure, all sections are immersed in 0.2 N HCl for 20 minutes, heat treated at 70°C in 2 x saline-sodium citrate (2 x SSC or 0.3 M NaCl plus 0.03 M trisodium citrate, pH 7.4) for 30
minutes, and digested with 1 μg/ml of proteinase K in a 2 mM CaCl₂, 10 mM Tris solution for 15 minutes at 37°C. In addition to sections processed in this way, we prepared sections omitting the 70°C heat treatment step. Sections to be examined for viral RNA were dehydrated in graded ethanol solutions and stored desiccated. Those to be examined for viral DNA were further rinsed in 2 x SSC, treated with RNAse A (100 μg/ml in 2 x SSC) for 30 minutes at 37°C, postfixed in 4% paraformaldehyde for 2 hr, dehydrated in graded ethanol solutions, and then stored desiccated.

All pretreated sections were subjected to denaturation by heat at 65°C in deionized formamide with 0.1 x SSC for 15 min, followed by quenching in ice-cold 0.1 x SSC, rinsing in water, and dehydration in graded ethanol solutions (10).

Sections were hybridized for 72 hr at 45°C with 35 μl of probe mixture per slide. The mixture contained approximately 10⁵ counts/min of ³⁵S-labeled PRV DNA in 2 x SSC with 10 mM Tris, 1 mM EDTA, 45% deionized formamide, 10% dextran sulfate, 0.35 mg/ml of murine L-cell nucleic acids, and 0.02% each of Ficoll (Pharmacia, Piscataway, NJ), polyvinyl pyrrolidone, and bovine serum albumin. Cover slips laid gently upon the sections were rimmed with rubber cement to prevent evaporation. After hybridization, the rubber cement-sealed cover slips were removed and the sections were washed for 48 hr in large volumes of 2 x SSC with 10 mM Tris, pH 7.4, 1 mM EDTA, and 45% deionized formamide (10). Sections were then washed twice in 2 x SSC and dehydrated in graded ethanol solutions.

For autoradiography, slides were coated with Kodak NTB-2
emulsion (6) diluted 1:1 in 0.6 M ammonium acetate. After exposure in complete darkness for 6 days at 4°C, they were developed in D-19 (Eastman Kodak, Rochester, NY) and fixed in Kodak fixer. The slides were then washed in water and stained with Mayer's hematoxylin and eosin.

Replicate sections from all the tissue blocks used for in situ hybridization were also prepared for routine histology and stained with Mayer's hematoxylin and eosin.
RESULTS

Alternative Fixatives

A number of routine fixatives that can handle whole or halved bovine and porcine brains were tentatively tested before settling on the small group of fixatives actually evaluated. These preliminary experiments revealed that strictly coagulative fixatives such as 70% acetone permitted in situ hybridization. Nonetheless, the resulting morphology was so poor that the use of acetone as a fixative was of limited value. Other preliminary work established that in situ hybridization was compatible with complex balanced fixatives such as modifications of Bouin's fluid. Elimination of picric acid from tissue sections fixed in modified Bouin's fluid, however, was unreliable and very time consuming. Only the three simple balanced fixatives previously listed, MOCA, FOAL, and BAFI, were therefore chosen for more detailed evaluation, in addition to PLP and formalin.

Section Retention or Survivability

To pathologists, an important consideration in selecting a tissue fixative for in situ hybridization is its ability to modify cellular components so as to keep tissue sections attached to glass throughout the entire required processing procedure. In our work, survivability, or the capacity of sections to be retained on the glass, ranged from no tissue loss to complete loss of tissues, with many intermediate gradations. Table 2 illustrates the proportion of slides that lost all the tissue and the proportion that lost none following pretreatment. Losses were
Table 2. Tissue losses and morphology of hybridized tissue sections

<table>
<thead>
<tr>
<th>Tissue fixative</th>
<th>Proportion of slides with loss of all tissues</th>
<th>Proportion of slides with no tissue losses</th>
<th>Typical cellular morphology after hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treated with heat</td>
<td>Pre-treated without heat</td>
<td>Typical cellular morphology after hybridization</td>
</tr>
<tr>
<td>MOCA</td>
<td>0/12</td>
<td>0/12</td>
<td>Good (some very good)</td>
</tr>
<tr>
<td>BAFI</td>
<td>2/12</td>
<td>0/12</td>
<td>Good (some fair)</td>
</tr>
<tr>
<td>FOAL</td>
<td>6/12</td>
<td>1/12</td>
<td>Good (some fair)</td>
</tr>
<tr>
<td>FORM</td>
<td>13/24</td>
<td>5/24</td>
<td>Fair (some poor)</td>
</tr>
<tr>
<td>PLP</td>
<td>11/24</td>
<td>9/24</td>
<td>Good (some very good)</td>
</tr>
</tbody>
</table>
lowest for MOCA fixative, while all the slides bearing formalin- or PLP-fixed tissues suffered at least partial loss.

Tissue losses were greatest for fixatives that cross-link proteins, such as formalin, even when the 70°C heat pretreatment step was omitted. To partially compensate for such losses, twice as many slides were prepared with formalin and PLP-fixed tissue blocks as with blocks from other fixatives. Taking into account partial tissue losses in addition to data from Table 2, the overall survivability of sections fixed in various ways was approximately as follows: MOCA 85%, BAFI 74%, FOAL 42%, formalin 39%, and PLP 29%. Visual inspection was used to identify slides with partial tissue retention.

Cellular Morphology

Cellular morphology after in situ hybridization was assessed by comparing nuclear fading, preservation of cell outlines, and recognizability of cell types in untreated control sections versus controls subjected to the entire in situ hybridization procedure. Fixatives deemed to produce the best and worst results are illustrated in Figure 1; note that adequate cellular morphology was observed in both cases; although tears, fractures, and folds plagued tissues fixed non-coagulatively. Omission of 70°C heat pretreatment improved morphology, but not appreciably. In Table 2, we provide a qualitative assessment of morphology results.
Figure 1. Adequacy of cellular morphology depending on the type of fixation, as observed in control tissues (A, judged best, fixed in PLP). Top, before in situ hybridization. Middle, 70°C heat-pretreated hybridized tissues. Bottom, hybridized tissues pretreated without 70°C heat. Actual magnification x395. Bar = 20 μm
Figure 1. (continued)
Adequacy of cellular morphology depending on the type of fixation, as observed in control tissues (B, judged worst, fixed in formalin). Top, before in situ hybridization. Middle, 70°C heat-pretreated hybridized tissues. Bottom, hybridized tissues pretreated without 70°C heat. Actual magnification x395. Bar = 20 μm
Compatibility with in Situ Hybridization

As shown in Table 3, all five fixatives permitted specific in situ hybridization. Relative grain densities were categorized from negative (no greater than background) to strong specific hybridization (+++). A grain density distinctly higher than background was categorized as (+) and was taken as an indication of positive results. Sections from 13 of the 15 infected animals featured various degrees of specific hybridization, whereas none of the controls yielded labeled tissues. Labeled cells were seen in linear arrangement at the injection sites. In addition, ependymal cells of the lateral ventricles and of the third ventricle as well as a number of hippocampal neurons were strongly positive. Figures 2 and 3 illustrate the heaviest grain densities observed in sections obtained through each of the fixation methods. Omission of 70°C heat pretreatment neither prevented strong specific in situ hybridization nor affected its intensity (Figure 3); it did, however, help to enhance section retention for tissues fixed with FOAL or formalin, and to a lesser extent for those fixed with PLP (Table 2).
Table 3. Effect of fixation on in situ hybridization^a

<table>
<thead>
<tr>
<th>Tissue fixative</th>
<th>Proportion of slides with strong (+++) hybridization</th>
<th>Proportion of slides positive with (+ to ++++) hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreated with heat</td>
<td>Pretreated without heat</td>
</tr>
<tr>
<td>MOCA</td>
<td>4/6</td>
<td>5/6</td>
</tr>
<tr>
<td>BAFI</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>FOAL</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>FORM</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td>PLP</td>
<td>3/7</td>
<td>4/7</td>
</tr>
</tbody>
</table>

^aSections from PRV infected animals only; uninfected controls featured no hybridization.
Figure 2. Strong specific in situ hybridization (+++) in differently fixed tissues. (A) MOCA; (B) BAFI; (C) FOAL; (D) PLP. Actual magnification x395. Bar = 20 μm
Figure 3. Specific in situ hybridization in formalin-fixed tissues. Top: 70°C heat pretreated, examined for DNA (A) and RNA (B). Bottom: 70°C heat pretreatment omitted, examined for DNA (C) and RNA (D). Actual magnification x395. Bar = 20 μm
DISCUSSION

An ideal fixative for in situ hybridization should meet the following criteria: First, it must be compatible with all steps in the procedure, preserving tissues nearly intact to the end; second, it must permit specific nucleic acid hybridization, so that a high grain density will develop against a low background; and third, it must preserve enough morphological detail to permit the localization of grain clusters to individual cells. By these criteria, none of the fixatives tested was a complete failure, but there were distinct differences between them. Because we only examined murine brain tissue, our results should be extrapolated to other species and to other types of tissue with caution. Moreover, the possibility of variation introduced by differences in laboratory routines for the preparation of paraffin blocks cannot be ruled out.

In terms of section retention, MOCA fixative fared best and the strictly noncoagulative fixatives, formalin and PLP, fared worst. Perhaps the cross-linking of proteins can firm up bonds within the tissue to the point that if any portion of a section loosens from the glass, the whole section is at risk. If so, then a possible solution is to improve uniformity of attachment to the glass using methods other than picking up free-floating ribbon sections from a water bath.

Cellular morphology after hybridization varied; consistently good results were obtained with MOCA and PLP, whereas formalin-fixed tissues often fared poorly. MOCA fixative penetrates well, rapidly firming up
the tissue, and its acetic acid counters much of the shrinkage that can be caused by ethanol. However, MOCA tends to disrupt erythrocytes and cytoplasmic organelles despite its formaldehyde content. PLP preserved cytoplasmic detail more effectively, but it penetrates slowly and it will not firm tissues appreciably; these are major drawbacks when attempting to fix a large brain in such a way as to retain proper three-dimensional relationships of its tissues during trimming.

The significance of variation in the proportion of positive sections obtained with each fixative (Table 3) could not be assessed because tissue losses greatly reduced the number of sections available in some categories. Moreover, different sections from six of the infected animals varied all the way from negative to strongly hybridized, indicating that not all parts of an infected brain were equally seeded with virus. Failure to observe hybridization in two of the infected animals was due to severe tissue section losses rather than to fixative-induced alterations in specificity, since analogously fixed tissues from other infected animals were positive.

In our search for fast-acting fixatives suitable for bulky brains, an incidental but important observation was that formalin fixation permits in situ hybridization (Figure 3). However, tissue losses and poor morphology suggest that use of formalin necessitates further modifications of pretreatment steps. Instead of, or in addition to, deleting 70°C heat treatment, it may be possible to alter other pretreatment steps without sacrificing the sensitivity of in situ hybridization. Retroactive studies using routinely processed blocks
are thus conceivable and should be attempted.

The best tissue fixatives in this study were deemed to be MOCA and PLP, and their use is recommended. The former yielded sections with mostly good morphology and minimal tissue loss during processing. The latter yielded sections rich enough in cytoplasmic detail to suggest that it merits serious consideration for use despite possible tissue losses. Losses of PLP-fixed tissues can undoubtedly be reduced by modifying pretreatment steps.
LITERATURE CITED


10. Stroop WG, Rock DL, Fraser NW: Localization of Herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by in situ hybridization. Lab Invest 51:27, 1984
ACKNOWLEDGMENTS

The authors thank Mr. Robert O. Ross for preparing the tissue blocks and for staining all the sections; Ms. Judith G. Wheeler for excellent technical assistance; and Dr. Pamela K. McAllister for developing and mounting the photographs.
SECTION III. REPRODUCIBILITY OF IN SITU HYBRIDIZATION
WHEN APPLIED TO MOCA AND FORMALIN-FIXED
PSEUDORABIES-INFECTED CATTLE TISSUES
Reproducibility of in situ hybridization when applied to MOCA and formalin-fixed pseudorabies-infected cattle tissue

Harold A. McAllister and Wayne A. Hagemoser

From the Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011

ABSTRACT

Both neural and non-neural tissues were collected from calves intranasally infected with aerosolized pseudorabies virus (PRV) and from control animals. A modification of Carnoy's fluid, MOCA fixative, was applied to them, then in situ hybridization was carried out to detect viral nucleic acids with $^{35}$S-labeled PRV DNA probes. The procedure was evaluated for reproducibility and degree of correspondence to quantitative virus isolation. Seven year old paraffinized tissue blocks of formalin-fixed neural material from an experimentally PRV-infected calf were concurrently examined. Reliable specific in situ hybridization occurred in neural tissues, but suspicious, seemingly false positive reactions were not uncommon in other tissues, particularly those rich in lymphocytes and macrophages. A strong hybridization signal was present in the formalin-fixed material despite its age.

KEY WORDS: Cattle, experimentally infected with pseudorabies virus; Modified Carnoy's (MOCA) and formaldehyde fixatives; Nucleic acid hybridization, in situ, with $^{35}$S-labeled DNA probes.
INTRODUCTION

In situ hybridization uses a complementary nucleotide sequence or "probe" that is either radioactively labeled or bound chemically to a "reporter" molecule (10). The probe is annealed to a nucleic acid of interest (a gene, messenger RNA or viral nucleic acid) and the resulting hybrids are detected by autoradiography, fluorescence, affinity chromatography or immunohistochemical means (4, 10).

Probes prepared by nick translation of viral nucleic acids have proven sensitive, specific, and sufficiently stable for new approaches in the study of viral infections (10). In situ hybridization of paraffin embedded material detects viral nucleic acid rather than antigens and preserves tissue morphology well. Methodology that avoids radioactive labeling is adequate for the detection of reiterated genes, but it is not considered very sensitive (4, 10).

After a radioactive probe is specifically bound and excess is washed off, its ionizing radiation can expose a photographic emulsion. The number of silver grains that develop at a hybridization site is roughly proportional to the number of probe copies bound (4, 10). Virus infected cells are then recognizable due to silver grain accumulations that exceed background.

Recent reports (10, 14, 15) examine the compatibility of various fixatives with in situ hybridization and compare their usefulness when applied in conjunction with that technique. A modification of Carnoy's fluid, MOCA, penetrates bulky masses of tissue rapidly and minimizes
the detachment of tissue sections from glass during hybridization (14). Although MOCA contains some formaldehyde, it is primarily a coagulative ethanol-acetic acid mixture. Haase et al. (10) reported that such a mixture optimally enhances hybridization. The efficiency of hybridization is lowered by cross-linking agents such as formalin (10), but as the most widely used fixative it is unavoidably involved in retrospective studies and is therefore of interest.

We chose to evaluate the reproducibility and effectiveness of in situ hybridization of MOCA-fixed tissues from cattle experimentally infected with pseudorabies virus (PRV) because of its high potential for the study of pathogenesis. In a previous report (14), we dealt with the application of the technique to murine neural tissues; here, results in a wide range of both neural and non-neural tissues are reported. In addition, we have looked retroactively at formalin-fixed neural sections from a PRV-infected calf.
MATERIALS AND METHODS

Infection of Animals

Sheep origin PRV strain VDL 2294 was secured frozen as a 2 ml suspension with 1 to 2 x 10^7 PFU/ml. Thawed PRV was aerosolized with a nebulizer moving air at 8 liters/minute (l/min) and producing 2.5 to 4.5 μm droplets. A heavy gauge plastic bag was placed over the calf's muzzle, sealed with tape, and filled with the infective mist for 5 to 8 min until the inoculum was consumed.

In the MOCA study, 2 PRV-infected (P3,P4) and 2 clinically normal control (C1,C2) calves were used. P3 and P4 weighed 90 to 115 kg; C1 and C2, 225 to 500 kg. All were electrocuted after the intravenous administration of xylazine. Infected calves were individually housed in isolation units. Controls, housed elsewhere, remained clinically normal. When killed 6 days (d) post-inoculation (PI), P3 had only early clinical signs of disease (anorexia, ataxia, high respiratory rate), while P4 had signs of being near death (severe ataxia, lethargy, head pressing, high temperature, labored respiration and intermittent lateral recumbency). The study of formalin-fixed materials was done on tissues from a calf (P5) that was inoculated by aerosol inhalation and killed moribund at 7 d PI seven years before the current work.

Fixatives

McAllister and Rock (14) previously described the preparation of MOCA and neutral buffered formalin-saline.
MOCA Fixation

Tissues, including the right or left half of the brain, were fixed in 4 l of MOCA for 16 hr. Trimmed tissues were transferred to 100% ethanol (EtOH) for 30 min and partially rehydrated by successive 30 min washes in graded alcohols (95, 85, and 80% EtOH). Final storage of tissues prior to routine dehydration, clearing, paraffin infiltration and embedding was in clean 70% EtOH.

Collection of Tissues for PRV Isolation

Using flame sterilized instruments, tissues for virus isolation (VI) were placed in sterile NASCO Whirl Pak™ bags (Fisher Scientific) and temporarily stored on dry ice. The half brain for VI was aseptically subdivided into blocks from specific anatomical sites. The blocks were then transferred to individual bags. All tissues were stored at -70°C until further processing.

Preparation of Tissue Cultures

Bovine lung cell monolayers, strain VMRI 219T, were trypsinized and resuspended in Eagle's minimal essential medium (7) with antibiotics (MEM-a) and 10% fetal calf serum. Linbro™ microtiter plates (Flow Labs, McLean, VA) or 4-chambered Lab-Tek™ slides (Miles Labs, Napierville, IL) were seeded with the suspension. Monolayers were grown, cells in 3 chambers were inoculated with 0.1 ml of a 10^-5 dilution containing 10 tissue culture infective doses 50 (TCID 50) of Becker PRV (14), and the cultures were incubated until typical cytopathogenic effect (CPE) plaques developed. Chamber slide monolayers fixed in acetone were used
as procedure controls in hybridization experiments while microtiter plate cultures were used in VI assays.

**Virus Isolation Assays**

About 1 g of each tissue was thawed and ground aseptically to produce a 10% suspension in MEM-a. The ice cold material was centrifuged at 600 x g for 10 min, and from the supernatant 10⁻¹ to 10⁻⁶ dilutions in MEM-a were prepared. Cultures were made by adding to each well in a row of a microtiter plate 0.05 ml of one of the dilutions, 0.05 ml of MEM-a, and 0.05 ml of a bovine lung cell suspension. These plate cultures were incubated 7 d with daily examination for PRV CPE. Reed-Muench (16) calculations were used to establish the number of TCID₅₀ per gram of tissue. No VI was attempted on tissues from Cl, C2, or P5. All VI assays were carried out within a week after collecting the tissues.

**In Situ Hybridization**

Briefly, this procedure entails the following: preparing a radioactive probe, chemically treating the glass slides, generating tissue sections, pretreating them, denaturing tissue nucleic acids, hybridizing tissue sections with the diluted working probe, autoradiography, and staining. A previously reported modification (14) of Brahic and Haase's procedure (3) was used except for additional changes noted below. Every test batch included both neural and non-neural tissues and positive and negative controls. Most hybridizations were done with extended storage of tissue blocks, 1 to 2 years after MOCA fixation and embedding.

A total genome probe (14) was used in the earliest work. It was
replaced by $^{35}$S-labeled, cloned PRV DNA Bam HI fragment 10' (12) probes of equal sensitivity and greater specificity and all tissues were reexamined with them.

Tissue blocks were embedded in Paraplast™ (Monoject Scientific, Sherwood Med. Industries, St. Louis, MO), ice cooled, and cut with disposable Accu-Edge™ (Lab-Tek, Napierville, IL) microtome knives at 5 μm thickness. Sections were collected on Denhardt-coated acetylated slides (14) by flotation in water at 41°C containing 0.1% Elmer's white glue (Borden, Columbus, OH). After overnight drying at room temperature, the sections were dry-heated horizontally at 60°C for 45 min. This heating step was repeated if storage for more than a few days intervened.

Pretreatment was done as before (14) except that the wet 70°C heating step was completely abandoned. Tissues to be examined for viral DNA were postfixed for 1.5 hr in fresh 4% paraformaldehyde; postfixation was omitted when examining for both DNA and RNA. Pretreated sections were dry heated for 15 min at 60°C before denaturation.

The volume of working probe mixture was increased from 36 to 60 μl to help minimize tissue detachment. An activity of $10^5$ counts/min (cpm) per slide was still sought, but the measured activity ranged from $4 \times 10^4$ to $1.5 \times 10^5$ cpm without detriment to results. Murine L-cell nucleic acids were replaced with equal parts of calf thymus DNA (Pharmacia, Piscataway, NJ) and calf liver RNA (Sigma, St. Louis, MO). Solutions of the two acids were separately sonicated, mixed, and kept frozen until used.

A safelight covered by a Kodak no. 2 filter (Eastman Kodak,
Rochester, NY) was used during slide dipping and during development of the emulsion-coated slides. Developed slides were stained by the Harris H&E procedure using freshly filtered stains.

Evaluation of Hybridization

A background of 15 grains or less per uninfected cell was considered acceptable. Grain densities below background were categorized as negative. A group of 40 to 60 clustered silver grains was deemed a (+) result. Over 60 grains clustered upon a still identifiable cell were recorded as (++)+. A cluster of grains too numerous to count which obscured underlying cells was classified as a (+++*) result (strong specific hybridization). The number of (+) to (+++) events was counted manually by visual inspection on a per section basis. Occasional (+) foci were disregarded as artifacts unless more than 3 were found.

Morphometric Analysis

Sections were projected on paper at 13.7 x magnification with a Bausch & Lomb microprojector and the tissue outline was drawn. A known length standard was also projected and drawn, then a Carl Zeiss Image Processing system (Carl Zeiss, Thornwood, NY) was used to determine each section's area in cm². At least two replicate sections per tissue block were used whenever possible. Finally, the number of hybridizing cells per unit area was determined in order to quantitatively compare VI and hybridization results.
RESULTS

Necropsy and Histopathology Findings

Control calves lacked gross lesions. Scattered lymphoid cell aggregates in the abomasal mucosa represented the only histopathologic finding in C1. Only minor microscopic lesions were present in C2, namely, a mild interstitial pneumonia, scattered myocardial sarcocysts, some lymphoid cell aggregates in the trachael mucosa, rare microfoci of hepatic necrosis, and a few renal interstitial lymphoid cell clusters.

All 3 PRV-infected calves were normal for 4 to 5 days, with rapid clinical deterioration on days 6 to 7 PI. Gross lesions were nonetheless absent in P3 and P5, and the only gross lesion in P4 was an atelectatic and multifocally hemorrhagic cardiac lung lobe.

Microscopic lesions in P3 were restricted to the stellate ganglion, in which there was moderate to severe lymphocytic infiltration, and to the trigeminal ganglion, which bore numerous leukocytes in blood vessels but lacked any signs of ganglioneuritis. Histopathologic lesions were essentially restricted to the central nervous system and the major ganglia in calves P4 and P5. The minor extraneural lesions noted in these animals were: some splenic lymphoid depletion, mild multifocal suppurative bronchopneumonia (P4 only), mild diffuse lymphocytic infiltration of turbinate and tracheal proprial tissue, and diffusely increased lymphoid cells in the esophageal muscularis.

Severe, widespread neural microscopic lesions in P4 and P5 were most prevalent in the cerebral cortex and the basal nuclei (Figures 1A, 1B). Involvement of the hippocampus, thalamus, mesencephalon, and cerebellum
Figure 1. Tissues hybridized in situ with a PRV DNA probe. H&E stain.
A, B: Perivascular cuff and glial nodule (arrow), respectively, in basal nuclei of infected calf P4, with strongly labeled cells (+++) near the lesions;
C: strongly labeled cells rimming CPE focus of PRV-infected tissue culture monolayer;
D: melanocytes mimicking labeled cells due to cytoplasmic granularity.
Actual magnifications: A, x175; B & C, x87; D, x345. Bar = 80 μm
was also considerable, with lesions decreasing in number and size caudally in the brain stem and spinal cord. Both gray and white matter were randomly involved. Among the lesions were multifocal lymphocytic meningeal infiltrates, perivascular lymphoid cell cuffs, areas of diffuse gliosis, scattered glial nodules, microfoci of necrosis, pyknotic glial cells, single partially or totally degenerated neurons, reactive astrocytes, and locally pronounced subependymal lymphocytic infiltrates. Variable numbers of lymphocytes were present in spinal ganglia and in the petrosal ganglion. The trigeminal ganglion was free of lesions in P5, but in P4 it had clusters of lymphocytes and increased numbers of capsular or satellite cells. In the stellate ganglion, there was only a mild diffuse increase in interstitial lymphocytes.

Extent of Hybridization in Neural Tissues
There was consistently reproducible labeling in all neural tissues expected to bear PRV (Table 1) and the number of cells counted was generally high. Variability in number was unrelated to the age of tissue blocks. Two year old blocks yielded replicate sections with as many or more labeled cells as had been counted in the earliest experiments, and all P5 blocks were 7 years old when used. Cells labeled as heavily as those in the neural tissues were only present around the CPE foci of PRV infected tissue culture monolayer controls (Figure 1C).

Effect of Postfixation on the Hybridization Signal
Since the number of labeled cells was lower than expected in all sections tested for PRV DNA (Table 1), 4 replicates of 3 different known
Table 1. Number of labeled cells per tissue section

<table>
<thead>
<tr>
<th>Consistently positive tissues examined for:</th>
<th>Range: Viral RNA+DNA</th>
<th>Range: Viral DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3 calf: PRV infected, MOCA fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stellate ganglion</td>
<td>60-80</td>
<td>0-30</td>
</tr>
<tr>
<td>P4 calf: PRV infected, MOCA fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>olfactory bulb</td>
<td>20-40</td>
<td>5-20</td>
</tr>
<tr>
<td>frontal cortex</td>
<td>300-1,000</td>
<td>20-150</td>
</tr>
<tr>
<td>basal nuclei</td>
<td>500-2,500</td>
<td>125-1,100</td>
</tr>
<tr>
<td>temporal cortex</td>
<td>60-450</td>
<td>15-250</td>
</tr>
<tr>
<td>thalamus</td>
<td>200-500</td>
<td>50-200</td>
</tr>
<tr>
<td>hippocampus</td>
<td>10-20</td>
<td>3-10</td>
</tr>
<tr>
<td>occipital cortex</td>
<td>100-250</td>
<td>15-80</td>
</tr>
<tr>
<td>cerebellum</td>
<td>100-200</td>
<td>10-70</td>
</tr>
<tr>
<td>mesencephalon</td>
<td>100-150</td>
<td>10-60</td>
</tr>
<tr>
<td>metencephalon (pons area)</td>
<td>80-120</td>
<td>3-10</td>
</tr>
<tr>
<td>anterior medulla</td>
<td>40-100</td>
<td>0-35</td>
</tr>
<tr>
<td>middle medulla</td>
<td>20-70</td>
<td>0-10</td>
</tr>
<tr>
<td>posterior medulla</td>
<td>15-35</td>
<td>0-10</td>
</tr>
<tr>
<td>spinal cord</td>
<td>10-150</td>
<td>1-10</td>
</tr>
<tr>
<td>P5 calf: PRV infected, formalin fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>frontal cortex</td>
<td>35-80</td>
<td>ND^b</td>
</tr>
<tr>
<td>parietal cortex</td>
<td>10-30</td>
<td>ND</td>
</tr>
<tr>
<td>occipital cortex</td>
<td>130-2,600</td>
<td>ND</td>
</tr>
<tr>
<td>thalamus-hypothalamus</td>
<td>100-470</td>
<td>ND</td>
</tr>
</tbody>
</table>

^aVariable numbers of replicate sections per tissue.

^bNot done.
positive tissues were chosen for simultaneous processing with and without postfixation. The 12 sections were exposed to the same probe mixture under virtually identical conditions. Paraformaldehyde postfixation was done only on 3, all examined for DNA, and they yielded only 23, 45 and 360 labeled cells, respectively. In contrast, the number of labeled cells per section ranged from 150 to 1,100 in 6 sections examined for DNA omitting postfixation. The remaining sections were examined for both RNA and DNA omitting postfixation and the total numbers of cells detected were 810, 1,300, and 1,600.

Reproducibility of Hybridization in MOCA-Fixed Tissues

Neural tissue results were consistent from one section replicate to another (Table 2). In non-neural tissues, however, there were instances suggestive of false positive or spurious hybridization (Table 3), although all known artifacts were ignored. Artifacts include grain clumps deposited around particles of debris as well as the melanocytes of gangli- onic, adrenal, and meningeal connective tissues (Figure 1D). Most control tissues featuring suspicious labeling originated from calf C2.

Extent of Labeling in Control Tissues

The number of seemingly hybridized cells in positive C2 tissues ranged up to 1,300 per cm², but only occasional spleen sections were labeled that heavily. More often, several dozens or a few hundred labeled cells were present, and only in some of the section replicates. Tissues rich in lymphocytes and macrophages, such as those from the lymphoid organs, liver, and gastrointestinal tract were generally
Table 2. Reproducibility of hybridization in MOCA-fixed neural tissues

<table>
<thead>
<tr>
<th>Calf tissues</th>
<th>Hybridized sections/Total number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Uninfected:</th>
<th>PRV infected:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C1</td>
<td>C2</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td></td>
<td>0/4</td>
<td>0/6</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td>0/12</td>
<td>0/24</td>
</tr>
<tr>
<td>Basal nuclei</td>
<td></td>
<td>0/4</td>
<td>0/11</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td>0/8</td>
<td>0/6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Mesencephalon</td>
<td></td>
<td>0/2</td>
<td>0/5</td>
</tr>
<tr>
<td>Metencephalon</td>
<td></td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Medulla</td>
<td></td>
<td>0/16</td>
<td>0/16</td>
</tr>
<tr>
<td>Spinal cord</td>
<td></td>
<td>0/2</td>
<td>0/16</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td></td>
<td>0/7</td>
<td>0/6</td>
</tr>
<tr>
<td>Petrosal ganglion</td>
<td></td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td></td>
<td>0/5</td>
<td>0/3</td>
</tr>
<tr>
<td>Stellate ganglion</td>
<td></td>
<td>0/4</td>
<td>0/7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numerator: number of sections with labeled cells yielded by a particular tissue block; denominator: total number of sections in the tally (all satisfactory, with tissue loss nil or under 30%).

<sup>b</sup>Total listed includes some sections where tissue loss exceeded 30%.
Table 3. Reproducibility of hybridization in MOCA-fixed non-neural tissues

<table>
<thead>
<tr>
<th>Calf tissues</th>
<th>Hybridized sections/Total number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PRV infected:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>C2</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0/2</td>
<td>1/9</td>
</tr>
<tr>
<td>Hypophysis</td>
<td>1/6</td>
<td>5/9</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>0/12</td>
<td>16/39</td>
</tr>
<tr>
<td>Tonsil</td>
<td>0/4</td>
<td>6/13</td>
</tr>
<tr>
<td>Thymus</td>
<td>0/4</td>
<td>4/10</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/4</td>
<td>2/8</td>
</tr>
<tr>
<td>Nasal turbinates</td>
<td>0/4</td>
<td>4/7</td>
</tr>
<tr>
<td>Tracheal mucosa</td>
<td>0/4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3/20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung</td>
<td>0/4</td>
<td>2/7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart</td>
<td>0/4</td>
<td>0/5</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Reticulorumen</td>
<td>0/4</td>
<td>1/6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Abomasum</td>
<td>0/4</td>
<td>2/7</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0/8</td>
<td>2/11</td>
</tr>
<tr>
<td>Spiral colon</td>
<td>0/4</td>
<td>2/9</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/2</td>
<td>4/10</td>
</tr>
<tr>
<td>Liver</td>
<td>0/4</td>
<td>4/7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numerator: number of sections with labeled cells yielded by a particular tissue block; denominator: total number of sections in the tally (all satisfactory, with tissue loss nil or under 30%).

<sup>b</sup>Total listed includes some sections where tissue loss exceeded 30%.
Figure 2. Tissues hybridized in situ with a PRV DNA probe. H&E stain.
A: Labeled cells, bronchial lymph node, control C2;
B: strongly labeled (+++) cells, formalin-fixed occipital cortex, infected calf P5;
C: tracheal mucosa with labeled cells, infected calf P4;
D: stellate ganglion, infected calf P3, featuring dense lymphocytic infiltration and labeled neurons.
Actual magnifications: A&D, x345; B&C, x175. Bar = 50 μm
Reproducibility of Hybridization in Formalin-Fixed Tissues

Slides from formalin-fixed neural material were characterized by reproducible results and no tissue losses (Table 4). The most extensive and impressive hybridization signal was detected in the occipital cortex (Figure 2B), which had the highest number of labeled cells per unit area.

Virus Isolation and in Situ Hybridization Compared

A quantitative comparison of VI and in situ hybridization was carried out by correcting the latter for area. Since only two animals are dealt with, an absolute or highly significant correlation between VI values and the corresponding hybridization labeling values per tissue type was neither sought nor expected. For a particular tissue, a different half of the body or even different regions of the same structure were involved in each one of the two procedures, and local homogeneity or perfect bilateral symmetry in the distribution of virions are unlikely. Nonetheless, when applied to neural tissues, overall agreement between the two techniques was good: very limited labeling developed in tissues from the calf that yielded no PRV by VI, and there was substantial labeling in tissues from the calf that yielded large amounts of PRV (Table 5). Labeling was heaviest and virus was most abundant in the telencephalic structures of P4 (the moribund calf). Agreement between the two procedures was poor and labeling results were often inconsistent in non-neural tissues (Table 6). Range values offered in Tables 5 and 6 were generally derived from examining 2 to 5 tissue sections, but a few
Table 4. In situ hybridization in formalin-fixed neural tissues

<table>
<thead>
<tr>
<th>Calf tissues</th>
<th>Hybridized/Total(^a)</th>
<th>Range: minimum-maximum(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRV infected: P5</td>
<td>P5: labeled cells/cm(^2)</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>4/4</td>
<td>15-35</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>4/4</td>
<td>5-15</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>4/4</td>
<td>270-545</td>
</tr>
<tr>
<td>Thalamus/hypothalamus</td>
<td>10/10</td>
<td>25-115</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>3/4(^c)</td>
<td>0-5</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>Petrosal ganglion</td>
<td>2/4(^c)</td>
<td>0-1</td>
</tr>
</tbody>
</table>

\(^a\)Numerator: number of sections with labeled cells yielded by a particular tissue block; denominator: total number of sections in the tally (all satisfactory, with tissue loss nil or under 30%).

\(^b\)Variable numbers of replicate sections per tissue.

\(^c\)Total listed includes some sections where tissue loss exceeded 30%.
Table 5. Comparison of quantitative virus isolation and labeling per unit area

<table>
<thead>
<tr>
<th>PRV infected neural tissues</th>
<th>Calf P3</th>
<th>Calf P4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labeled cells/cm²</td>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;/g</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>0</td>
<td>45-95</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0</td>
<td>280-945</td>
</tr>
<tr>
<td>Basal nuclei</td>
<td>0</td>
<td>175-870</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>0</td>
<td>30-200</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0</td>
<td>70-180</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0</td>
<td>20-40</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>0</td>
<td>45-110</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0</td>
<td>55-115</td>
</tr>
<tr>
<td>Mesencephalon</td>
<td>0</td>
<td>50-75</td>
</tr>
<tr>
<td>Metencephalon</td>
<td>0</td>
<td>45-70</td>
</tr>
<tr>
<td>Anterior medulla</td>
<td>0</td>
<td>15-40</td>
</tr>
<tr>
<td>Middle medulla</td>
<td>0</td>
<td>15-45</td>
</tr>
<tr>
<td>Posterior medulla</td>
<td>0</td>
<td>15-30</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0</td>
<td>10-145</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>0-35</td>
<td>0</td>
</tr>
<tr>
<td>Petrosal ganglion</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>0</td>
<td>0-60</td>
</tr>
<tr>
<td>Stellate ganglion</td>
<td>165-220</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Range: minimum to maximum number recorded.

<sup>b</sup>Tissue culture infective dose<sub>50</sub> per gram of tissue.

<sup>c</sup>Not done.
Table 6. Comparison of quantitative virus isolation and labeling per unit area

<table>
<thead>
<tr>
<th>PRV infected non-neural tissues</th>
<th>Calf P3</th>
<th></th>
<th>Calf P4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labeled</td>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;/g&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Labeled</td>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;/g&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nasal turbinates</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>744</td>
</tr>
<tr>
<td>Tracheal mucosa</td>
<td>0</td>
<td>0</td>
<td>0-825</td>
<td>429,000</td>
</tr>
<tr>
<td>Lung</td>
<td>0-5</td>
<td>0</td>
<td>0-145</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>0</td>
<td>0-240</td>
<td>0</td>
</tr>
<tr>
<td>Tonsil</td>
<td>0-85</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thymus</td>
<td>90-700</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50-470</td>
<td>0</td>
</tr>
<tr>
<td>Retropharyngeal l.n.</td>
<td>0-35</td>
<td>0</td>
<td>0-55</td>
<td>0</td>
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<tr>
<td>Bronchial lymph node</td>
<td>0-50</td>
<td>6,124</td>
<td>0-165</td>
<td>0</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>0-85</td>
<td>ND</td>
<td>0-280</td>
<td>0</td>
</tr>
<tr>
<td>Adrenal</td>
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<td>0</td>
<td>0-1</td>
<td>0</td>
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<tr>
<td>Hypophysis</td>
<td>0</td>
<td>ND</td>
<td>0-20</td>
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<tr>
<td>Kidney</td>
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<td>0</td>
</tr>
<tr>
<td>Liver</td>
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<td>0</td>
<td>0-60</td>
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<tr>
<td>Esophagus</td>
<td>0</td>
<td>0</td>
<td>0-8</td>
<td>8,140</td>
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<tr>
<td>Reticulorumen</td>
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<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
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<td>Abomasum</td>
<td>0</td>
<td>ND</td>
<td>120-350</td>
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<tr>
<td>Duodenum</td>
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<td>ND</td>
<td>350-1,200</td>
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<tr>
<td>Ileum</td>
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<td>ND</td>
<td>0-170</td>
<td>ND</td>
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<tr>
<td>Spiral colon</td>
<td>0</td>
<td>ND</td>
<td>0-425</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>Range: minimum to maximum number recorded.

<sup>b</sup>Tissue culture infective dose<sub>50</sub> per gram of tissue.

<sup>c</sup>Not done.
reflect results from 1 to 3 dozen replicates.

Surprisingly, large amounts of virus were recovered from the tracheal mucosa of P4 but not that of P3. Strongly labeled cells were present in the tracheal propria of P4 (Figure 2C). Heavily labeled cells were detectable only in the stellate ganglion of P3 (Figure 2D), the calf with early disease signs, and VI demonstrated PRV in only one of its bronchial lymph nodes. The possibility of false positive labeling in non-neural tissues casts doubt on the apparent detection of many hybridized cells in P3's thymus, tonsils, and lymph nodes.

Morphologic Lesions and in Situ Hybridization

The correlation between histopathologic neural lesions and cellular labeling was imperfect. Microfoci of necrosis, partially degenerated neurons, perivascular lymphoid cuffs and other obviously damaged sites often did not feature specific labeling. Moreover, neurons with label were not necessarily in stages of degeneration recognizable by light microscopy. This was evident from inspection of either lightly labeled ganglionic neurons or heavily labeled cerebral nuclei also identifiable in non-hybridized, routinely stained replicate tissue sections. Although damage without labeling and labeling without obvious damage were both apparent, lesions and labeling did tend to occur concurrently in a tissue.

Examination of Tissue Sections for Other Viruses

Replicate sections of renal, hepatic, lymph node, and thymic C2 tissues that were prone to take PRV label, as well as sections of thymic and bronchial lymph node tissues from P3 and P4 analogously prone to such
labeling were prepared. All failed to take up label when hybridized in situ with an Infectious Bovine Rhinotracheitis virus (IBRV) purified DNA probe.
DISCUSSION

A fulminating onset of clinical pseudorabies 6 to 7 days PI, characteristic for cattle, correlates well with explosive viral multiplication demonstrable by VI and in situ hybridization. Little or no virus might be detectable prior to that point. The virtual absence of questionable hybridization in numerous neural tissues, the extent and intensity of their labeling, and general agreement with neural VI results suggest that our methodology can be applied with some confidence to the study of PRV infection in the bovine nervous system. However, since there was inconsistent hybridization in non-neural C2, P3, and P4 tissues (Table 3), and since agreement between hybridization and VI in the corresponding P3 and P4 tissues was poor (Table 6), additional work is necessary before expanding use of the technique beyond the nervous system in the study of pseudorabies pathogenesis.

The inclusion of known positive and known negative material in every batch, the use of adequate numbers of control animals whenever feasible, continuous assessment of reproducibility, and the introduction of some methodological controls cannot be emphasized enough when dealing with in situ hybridization. The limited scope of our work precludes offering a definitive explanation for our problems with non-neural tissues, but some ideas can be explored.

Misleading cellular hybridization could conceivably stem from infection by a herpesvirus that, unlike PRV, can produce subclinical problems, such as IBRV. Several authors (1, 2, 6, 18) have shown
antigenic kinship between PRV and IBRV; others have found genomic similarities (5). Both C1 and C2, however, were free of IBRV antibodies, and 16 tissues prone to questionable PRV hybridization in our work failed to bind an IBRV DNA probe.

Nonspecific or false positive hybridization is an alternative possibility. Jones and Hyman (11) reported that *Herpes simplex* DNA and human DNA can bind due to guanine-rich polymers interacting with cytosine-rich tracks rather than through authentic base sequence homology. Singh and Jones (17) pointed out that binding of labeled DNA probes by the proteins of certain cells can occur, and they proposed the use of heparin to block it. Maitland et al. (13) have also reported nonspecific binding of nucleic acid probes by some cells, especially certain lymphocytes.

To fully exploit the sensitivity of in situ hybridization, a strong, undimmed signal is essential. In Table 1, we documented decreased hybridization in sections examined for DNA even though the RNase we used was reportedly monophoretic and without known DNase activity. To some extent, the decreased labeling should relate to the presence of less viral DNA than viral mRNA in infected cells. In addition, the efficiency of in situ hybridization to RNA is about 100% as opposed to only 20 or 30% for DNA (10). A third factor may be the 1.5 hr paraformaldehyde postfixation. Prolonged paraformaldehyde postfixation of sections to be examined for DNA was proposed by Haase et al. (10), who discovered that RNase digestion and subsequent denaturation partially solubilizes DNA, causing significant losses during hybridization. Tissues
suboptimally fixed with poorly penetrating agents might exacerbate the problem. In this context, it may be important to consider that formalin penetrates rapidly but fixes slowly (9). Paraformaldehyde postfixation stabilizes the DNA (10). Nonetheless, prolonged postfixation of material that is already well-fixed and reduced to a section a few micrometers thick might so thoroughly cross-link all macromolecules that it could negate to a degree the intent of tissue pretreatment, which is to facilitate probe diffusion into the cell. Decreased efficiency of hybridization has been reported when cells are exposed to paraformaldehyde or glutaraldehyde for extended periods (4, 10). It is not desirable or necessary to eliminate postfixation, but it should be shortened or its duration should be optimized according to the type of fixative used on the tissues.

Moench et al. (15) discovered that standard nick translation protocols generate probes that are too long for optimal penetration of tissues fixed by the cross-linking aldehydes. Such fixatives produce a proteinaceous network that hinders probe diffusion and impairs the annealing of bases (10). Investigators should, therefore, also consider adjusting probe length to the type of fixative used.

In conclusion, in situ hybridization of a wide range of MOCA-fixed, bulky, PRV-infected cattle tissues is practical, and retrospective research with formalin-fixed material is feasible. Additional refinement in methodology to eliminate inconsistency in non-neural results is necessary, and alternative approaches using biotin-labeled probes (4) and plastic embedding (8) should be pursued.
LITERATURE CITED


2. Aguilar-Setién A, Vandeputte J, Pastoret PP, Michaux C, Pensaert MB, Schoenaers F: Présence concomittante, chez les bovins et les porcs, d'anticorps neutralisant le virus de la rhinotrachéite infectieuse bovine (Bovid Herpesvirus 1, BHV 1) et celui de la maladie d'Aujeszky (Sus Herpesvirus 1, SHV 1), après contact avec le virus homologue. Ann Méd Vét 123:275, 1979


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GENERAL DISCUSSION AND SUMMARY

This thesis is presented in the alternate format and it is composed of three interrelated but independent sections or manuscripts.

Section I, titled "Pseudorabies in cattle: A review," is a critical rather than a historical review of a considerable proportion of the literature that is available on the subject. It focuses on the problem from the perspective of pathogenetic mechanisms. In addition, it highlights opportunities for additional research, raises questions about accepted views, and suggests that recently developed techniques may prove useful in gaining new insights about this particular infectious process.

Section II is titled "Comparative usefulness of tissue fixatives for in situ viral nucleic acid hybridization." It deals with the student's efforts to adapt one of the newer research techniques for its use in pseudorabies-infected cattle tissues. The work was actually carried out in mice in order to limit expenses and conserve resources. It established that the available hybridization probes could detect nucleic acids of pseudorabies virus effectively and specifically. The paper also offers a detailed account of the various fixatives tested for degree of usefulness and compatibility with in situ hybridization. A modification of Carnoy's fluid (MOCA) was devised and it was found to be well-suited for fixation of bulky cattle tissues.

Section III is titled "Reproducibility of in situ hybridization when applied to MOCA and formalin fixed pseudorabies-infected cattle tissues." It documents the reproducibility of in situ hybridization as
well as its overall correlation to quantitative virus isolation when it was applied to a wide range of MOCA-fixed, pseudorabies infected bovine tissues. In addition, the paper offers the results of a limited retroactive study carried out on seven year old, formalin-fixed, PRV-infected neural bovine tissues.

The experimental work reported in Section III represents a departure from an original intention to study the pathogenesis of bovine pseudorabies, by means of in situ hybridization, in tissues from calves infected by the inhalation of aerosolized PRV. The focus of the study shifted to the technique itself because under certain circumstances labeling could not be consistently reproduced.

Reliable specific in situ hybridization occurred in MOCA-fixed neural tissues, but suspicious, seemingly false positive reactions were not uncommon in other tissues, particularly those rich in lymphocytes and macrophages. A strong hybridization signal was present in the formalin-fixed neural tissues despite their age; results with this material were analogous to those obtained with MOCA-fixed tissues.

The shift in focus was also strongly supported by finding that in a calf with early signs of Aujeszky's disease, neither in situ hybridization nor quantitative virus isolation detected significant amounts of pseudorabies virus. More confidence in hybridization results, especially outside the nervous system, seemed imperative.

Improvements in methodology almost completely eliminated neural tissue losses with both MOCA-fixed and formalin-fixed materials, and they brought non-neural tissue losses down to 20% or less. Methodologic
refinements, however, did not eliminate the aforementioned questionable labeling, and ultimately only three pseudorabies-infected calves were critically examined. Moreover, in only two of those three calves, both neural and non-neural tissues were subjected to parallel in situ hybridization and quantitative virus isolation (no attempts to recover virus had been carried out on the 7 year old tissues).

The small number of experimentally infected calves involved in Section III and the technical limitations encountered precluded reaching any sweeping generalizations about the pathogenesis of bovine pseudorabies. Nonetheless, the section's results, as depicted in its tables, are compatible with a few cautious remarks that may add some insight to the nature of this disease.

In the first place, it seemed that prior to the onset of a full range of clinical signs very little viral replication occurred, and that this early replication was restricted to the stellate ganglion, to some lymph nodes, and, perhaps, to the thymus. Secondly, an explosive phase of viral replication was documented in moribund calves, and it included replication in the mucosa of the trachea and the esophagus. It might have also involved replication in the mucosa of the abomasum and the duodenum. Lastly, most of the explosive replicative activity in brain tissues occurred in telencephalic structures rather than in the brain stem. Viral replication was especially pronounced in the cortex, the basal nuclei, and parts of the hippocampus. These results are in agreement with some of the newer findings reported in the literature and discussed at considerable length in Section I of this thesis.
Since it would be intellectually dishonest to imply that Section III work supports any further thoughts pertaining to the pathogenesis of bovine pseudorabies, all ideas on this subject have been deliberately confined to Section I. It should be apparent to most readers that the author allowed his imagination to roam in that section. The reader is therefore invited to review Section I with this fact in mind.

A description of the development of tissue lesions does not suffice for us to understand the pathogenesis of a viral infection. To achieve a dynamic perception of the origin of viral disease, certainty about natural reservoirs, portals of entry, localization and replication of the agent, viral effects on the host, and the host's response to the infection are all essential and should be sought. As pointed out in the conclusion to Section I, we are far from having a complete and detailed understanding of the interaction between PRV and its bovine host.

Perhaps so little has been learned about pathogenetic mechanisms because few investigators have applied multidisciplinary approaches to the problem. To succeed in answering critical questions, the student of viral pathogenesis must know about the virus and its needs, about the nuances of the immune response and of species variability, about the chemistry and limitations of the available techniques for study, and about a multitude of other factors. Without collaboration with allied specialists, such as microbiologists and biochemists, all research efforts in this area may therefore be severely hampered. The acceptance of Veterinary Pathology as a purely descriptive endeavor is a notion that must be brought to an end.
GENERAL ACKNOWLEDGMENTS

I thank my major professor, Dr. W. A. Hagemoser, for having given me the opportunity to work with him in the field of bovine pseudorabies and for guiding me to our mutual friend and colleague, Dr. D. L. Rock, who made possible most of my research.

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Very special thanks are extended to Mrs. K. S. Pierce and to her
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