Comparison of Pseudorabies Virus Inactivated by Bromo-Ethylene-Imine, ^{60}Co Irradiation, and Acridine Dye in Immune Assay Systems†

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Pseudorabies virus infections among animals, especially swine, have become prevalent in the United States in the past few years. The disease in swine is now economically important. Test systems and antigens are being developed for use in control and disease suppression efforts. Pseudorabies virus was inactivated by three methods: chemically, with bromo-ethylene-imine; physically, with ^{60}Co irradiation; and chemically and physically, with 3,9-diaminoacridine dye followed by exposure to white visible light. The antigenicities of the preparations were determined in the presence of specific antibody in immunodiffusion tests and through immunoelectrophoresis. The latter technique permitted quantitation of either antigen or antibody. In the electrophoretic patterns, the antigenic mass in bromo-ethylene-imine preparations was estimated to be 42 mg/ml, the same as in the untreated control material. After ^{60}Co irradiation, 22 mg/ml was present, in comparison with 50 mg/ml in the untreated control antigen. In contrast, 67 mg/ml was present in the acridine dye-light-treated preparation, in comparison with 58 mg/ml in the untreated control material. A possible explanation for the acridine dye-light-treated preparation values is that photodynamic inactivation interferes with viral maturation during the replicative cycle within cells, with a resulting production of a greater amount of antigen, at least some of which is in the form of defective particles.

Pseudorabies virus (PrV) causes lethal infections in a broad spectrum of animal species. Swine, however, may survive infection and remain asymptomatic with latent infection. Under poorly understood circumstances some of these swine may shed the virus for unknown periods of time. Latent infection in which viral shedding occurs is evidenced mainly by the appearance of the disease in susceptible swine or other species. In rare cases this may be followed by isolation of the virus from oral secretions of the latently infected swine. The disease is now of much economic importance in the United States. As a consequence, a need has arisen for antigens to be used in diagnostic tests in the laboratory and in swine in the field.

Foot-and-mouth disease virus has been inactivated in cell culture fluids when exposed to a chemical agent, 2-bromo-ethylene-imine (BEI) (2, 3, 5, 18). Influenza and mumps viruses have been inactivated by physical means through gamma-particle irradiation from a ^{60}Co source

(14, 15). Exposure of herpes simplex virus to acridine dye and visible light resulted in inactivation of the virus (8, 13). These techniques have been used to destroy the infectivity and replicative potential of viruses while retaining most of the immunogenic properties.

This report presents results of the application of these three viral inactivation techniques to PrV. The antigens thus produced were evaluated qualitatively through an immunodiffusion test (IDT) and an electroimmunoassay system. Quantitation of antigens was possible in the immunoelectrophoretic test (IE), as the linear measure of movement or surface area of the precipitate is directly proportional to the amount of antigen (or antibody) present in the samples. These antigens have potential use for diagnostic tests and in the production of vaccines.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline consisted of 8.0 g of NaCl, 0.20 g of KCl, 1.15 g of Na_2HPO_4 , and 0.20 g of KH_2PO_4 per liter. Borate buffer was prepared by mixing 2.0 g of NaOH and 9.0 g of H_3BO_3 with 1

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liter of distilled water, pH 8.6. Tris-barbiturate buffer contained 22.4 g of sodium barbiturate, 44.3 g of tris(hydroxymethyl)aminomethane, 0.533 of calcium lactate, and 0.65 g of NaN_3 , pH 8.6, which was diluted 1:4 before use. The washing solution was 0.1 M NaCl solution. Staining solution was prepared by mixing 1 g of Coomassie brilliant blue R-250, 90 ml of ethanol, 20 ml of acetic acid, and 90 ml of distilled water. Destaining solution was the same as the staining solution but without Coomassie brilliant blue R-250.

Cells. Pig kidney cells (PK-W2E) derived from 3-week-old pigs were used at passage 70 in the studies. The cells were grown in flasks with Eagle medium containing 10% fetal bovine serum, 100 U of penicillin, and 170 mg of streptomycin per ml at pH 7.4 and maintained in a similar medium containing 2% fetal bovine serum.

Virus and infectivity assay. The FH and S strains of PrV were used throughout the study. Virus infectivity was assayed in PK cells by determination of the 50% tissue culture infective dose (TCID_{50}) at 48 h postinfection. Viral protein was determined by the biuret method (11) on samples previously subjected to sonic treatment and centrifuged. Highly purified bovine serum albumin was used as the standard.

2-Bromoethylamine hydrobromide inactivation. 2-Bromoethylamine hydrobromide (Eastman Kodak Co., Rochester, N.Y.) inactivant was cyclized as described previously (2, 3). The cyclized 2-bromoethylamine hydrobromide became BEI. The inactivation was effected by mixing PrV viral suspensions with BEI to a concentration of 0.001 M at 37°C for 6 h.

Physical treatment with ^{60}Co . Viral suspensions harvested from cell cultures and frozen at -90°C were placed in a ^{60}Co source chamber in a vessel containing dry ice (solid CO_2). Virus was exposed to a gamma flux of approximately 1.259×10^5 rads/h. Samples were withdrawn at various times for infectivity assays in cell culture.

Acridine dye-visible light irradiation. For acridine dye-visible light irradiation, the light source was essentially the same as that described previously (4). The procedure for photodynamic treatment was modified from the technique used previously (9, 19). Briefly, confluent PK-cell monolayers were infected at a multiplicity of infection of about 10 per cell. After absorption for 60 min at 34°C, the cell monolayers were washed twice with phosphate-buffered saline to remove nonabsorbed virus. 3,9-Diaminoacridine dye (acridine orange; Mead Johnson & Co., Evansville, Ind.) was then added, and incubation was continued for another 60 min in the dark. After incubation, the cells were irradiated with visible light for various period of time. The irradiated cells were then incubated in the dark for 24 h, after which the fluids containing the virus were harvested.

IDT. Identification of viral antigens in the test preparations was conducted in Noble agar (1% agar in borate buffer solution, pH 8.6) in petri dishes 35 mm in diameter. The plates were incubated in a humidified chamber at room temperature (24°C) and examined for the presence of an immunoprecipitation band periodically between 24 and 72 h.

A seven-well pattern was used, with one center well and six wells in a circle around it. The wells were 3

mm apart and 6 mm in diameter. Specific swine anti-PrV serum was placed in the center well. Alternate wells in the circle were filled with the test sample and the positive control sample. This arrangement provided a precipitin line on each side of the precipitin line of the tested sample, thus facilitating accurate determination of lines of identity.

IE. IE, modified from previous methods used by others (10, 21), was performed in agarose gel (1% agarose in Tris-barbiturate buffer, pH 8.6). The prepared antigens (25 μl) were simultaneously subjected to electrophoresis in 1% agarose containing either 2.5% normal pig serum or 2.5% swine anti-PrV serum. Immunoprecipitated protein was stained with 0.5% Coomassie brilliant blue R-250 as described previously (20). Nonprecipitated proteins were removed before staining by washing the gel plate three or more times in 0.1 M NaCl and distilled water. The plates were then dried in warm, dry air. Titration of antiserum was conducted similarly, using a constant amount of antigen (2.5%) mixed in the agarose.

FA technique. Fluorescent antibody (FA) studies were conducted by methods described in detail previously (12).

RESULTS

Chemical inactivation of PrV with BEI. The inactivation of PrV strains by BEI was a function of time (Fig. 1). With a constant amount of BEI, the titer of surviving virus detected in cell cultures steadily decreased in direct relation to the time of incubation. The inactivation of virtually all viral particles was attained after about 6 h of incubation (infective titer, $<1 \text{ TCID}_{50}/0.1 \text{ ml}$). Inactivated samples were tested for antigenic proteins by IDT and IE procedures.

FIG. 1. Inactivation of PrV suspensions (PrV-FH) by BEI. A constant amount of BEI (0.001 μl) was used. Samples were obtained at various times during the course of inactivation. Assay was performed on PK-W2E cells.

Physical inactivation of PrV through ^{60}Co irradiation. When the amounts of surviving viral fractions, as detected in cell cultures, were plotted on a logarithmic scale against time of irradiation, the points at which samples were withdrawn were found to lie on a straight line through 29 h. This indicated that the surviving fraction was an exponential function of the length of irradiation time. PrV samples were no longer infectious after 29 h of irradiation with a total dosage of 3.6511×10^6 rads (Fig. 2). Samples withdrawn after this treatment were evaluated in the two test systems (IDT and IE).

Chemical-physical inactivation of PrV with acridine dye and light. Previously reported work used acridine dye at a concentration of $5 \mu\text{g}/\text{ml}$ to inactivate virus (1, 22). We used acridine dye on PrV at a much lower concentration ($0.08 \mu\text{g}/\text{ml}$, as reported by others [8]). The kinetics of photodynamic inactivation of PrV-FH are illustrated in Fig. 3. The results presented here indicate that the irradiated virions lost their plaque-forming ability in a linear fashion during the replicative cycle. The infective titer of photodynamically treated virus was drastically reduced, to $<1 \text{ TCID}_{50}/0.1 \text{ ml}$, in 50 min, whereas no reduction in infectivity was observed in the control, which was exposed to light only. Others (4) have also found that inactivation does not occur in the presence of visible light or dye alone. After 40 min of exposure to the light, $0.08 \mu\text{g}$ of acridine per ml seemed to be as efficient as $5 \mu\text{g}/\text{ml}$ in protecting the cells from damage by the infectious virus PrV-S (Table 1).

FIG. 2. Survival curve of gamma-irradiated PrV-FH suspensions. Infectivity assays were performed at various times on PK-W2E cells. Exposure rate of gamma flux emitted was 1.259×10^5 rads/h.

FIG. 3. Kinetics of photodynamic inactivation of PrV-FH during its replicative cycle. Symbols: ●, PK-W2E infected cells treated with acridine dye ($0.08 \mu\text{g}/\text{ml}$) plus light irradiation for various time periods, as illustrated on the graph; ◆, control, PK-W2E infected cells treated with light only.

TABLE 1. Effect of acridine dye (AD) on viral host cell-lysing ability^a

Samples	OD ₅₄₀ ^b	Cells lysed (%)
Cell control (no virus, no AD)	0.55	0
Infected cell + 5 μg of AD/ml	0.45	33
Infected cell + 0.08 μg of AD/ml	0.47	27
Virus control (no AD)	0.25	100

^a PrV-S was grown in PK-W2E cells at multiplicity of infection of 10; exposure time was 40 min.

^b Optical density at 540 nm (OD₅₄₀) was taken immediately after harvesting the virus at 24 h postinfection.

A PrV cell-free suspension containing $10^6 \text{ TCID}_{50}/\text{ml}$ was exposed to the same regimen of inactivation as that for virus-infected cells. Controls consisted of the viral suspension exposed to light without dye and viral suspension incubated in the dark. At 24 h, all three samples were found to contain 10^6 TCID_{50} of virus per ml. This evidence supports the contention that photodynamic inactivation in the acridine dye system occurs during the replicative cycle of the virus in the cells.

Exposure time for photodynamic inactivation of PrV varied with the strain of PrV used. For maximum inactivation by acridine dye, PrV-FH required a single photodynamic treatment only, whereas PrV-S required triple challenges with light (Table 2). It is known that PrV-S is more

virulent than PrV-FH and that PrV-FH consistently elicits a higher virus-neutralizing antibody response in the serum of swine. The acridine dye regimen which resulted in the loss of ability to produce cytopathic effects on test cells was used to make antigens for evaluation by means of IDT and IE.

IDT to demonstrate antigens in inactive PrV preparations. An IDT analysis of BEI-treated antigen is presented in Fig. 4. Antiserum, in well 7, reacted with wells 2, 4, and 6 (filled with BEI-treated antigen) as it did with wells 1, 3, and 5 (filled with control positive antigen). The test antigen and control antigen showed

TABLE 2. Infectivity assay of virus produced in PK cells after single, double, and triple photodynamic treatments^a

Photodynamic treatment (h postinfection) ^b	Virus titer (TCID ₅₀ /ml) ^c
None (control)	10 ⁵
Single (2)	10 ²
Double (2, 4)	10
Triple (2, 4, 6)	<10

^a PrV-S was grown in PK-W2E cells.
^b Length of time for each photodynamic treatment was 60 min.
^c Virus was harvested at 24 h after exposure.

reactions of identity. Although IDT cannot be used to clearly quantitate antigenic proteins, it is a valuable qualitative test. However, an equilateral hexagonal ring of precipitate at least suggested that the antigenicity of the BEI-treated antigen was equal to that of the untreated positive control. ⁶⁰Co-irradiated antigen (Fig. 5, wells 2, 4, and 6) also gave positive results and formed a continuous line of identity with the control antigen. However, the antigenicity of this physically treated antigen seemed to be weaker than that of the reference control as shown in this IDT pattern. A weakly positive sample always resulted in a precipitin band near to the sample well, thus forming a distorted hexagonal ring (Fig. 5). Photodynamically prepared antigen gave a strongly positive test (Fig. 6). The stronger antigenicity of photodynamically inactivated antigen (wells 1, 3, and 5) in comparison with that of the positive control reference (wells 2, 4, and 6) also resulted in the formation of a nonsymmetrical hexagonal ring of precipitate. When normal pig serum was placed in the center well, lines of precipitation were not obtained, ruling out the possibility that immunoprecipitin bands in tests of antigens against antibody-containing sera were nonspecific reactions.

FIG. 4. Analysis of BEI-treated antigen by immunodiffusion. Equal volumes (40 μ l) of control positive antigen and test sample were present in the wells. Wells 1, 3, and 5 contained control positive antigen; wells 2, 4, and 6 contained BEI-treated antigen; well 7 contained anti-PrV swine serum.

FIG. 5. Analysis of ⁶⁰Co-irradiated antigen by IDT. Equal volumes (40 μ l) of control positive antigen and test sample were present in wells. Wells 1, 3, and 5 contained control positive antigen; wells 2, 4, and 6 contained ⁶⁰Co-irradiated antigen; well 7 contained anti-PrV swine serum.

FIG. 6. Analysis of photodynamically inactivated antigen by IDT. Equal volumes (40 μ l) of control positive antigen and test sample were present in the wells. Wells 2, 4, and 6 contained control positive antigen; wells 1, 3, and 5 contained photodynamically inactivated antigen; well 7 contained anti-PrV swine serum.

Quantitation of antigenic proteins with IE. Antigen from above preparations was quantitated by IE. Plots of immunoprecipitate distances of PrV standards from IE are shown in Fig. 7. BEI treatment did not reduce or destroy the ability of viral antigenic polypeptides to react with specific antibody (Fig. 8). Significant differences in immunoprecipitate mobilities were not observed between the untreated control antigen (B) and the BEI-treated antigen (C). Based on the linear span and compared with the PrV standard (A), a quantitative estimate of BEI-treated antigenic protein was 42 mg/ml. ^{60}Co -irradiated antigen (Fig. 9A) lost some ability to react with specific anti-PrV serum. From the immunoprecipitation pattern, the ratio of antigenic determinants between ^{60}Co -irradiated antigen and positive control was 22 mg/ml:50 mg/ml. This was at variance with another study (7), in which irradiated vaccinia virus antigenic determinants were completely destroyed.

In contrast to both chemically and physically prepared antigens, photodynamically inactivated antigen was found to have greater antigenicity than the untreated control (67 mg/ml:58 mg/ml) (Fig. 10). There is no evidence that photodynamic reaction plus acridine dye affects viral DNA synthesis (8). It is possible that the photodynamic technique affects certain processes during viral maturation, causing increased

FIG. 7. Plots of immunoprecipitate linear spans of PrV standards (30 mg/ml) from IE. IE was done on a glass plate (9.4 by 8.4 cm) in 1.5-mm-thick 1% (wt/vol) agarose gel. The gel was dissolved in a buffer as described in the text. IE was performed by applying 80 V of field strength for 15 h. (A) 0 λ , (B) 2 λ , (C) 6 λ , (D) 18 λ , (E) 54 λ .

production of immature or defective virions which retain antigenic determinants.

Quantitation of antiserum with IE. IE may also be used in the titration of antiserum. For a given antigen concentration, the relationship between the distance traveled by the precipitate and the antiserum titer is linear. Based on this principal, the titer of antiserum was evaluated. Results of IE and other diagnostic tests on several sera are compared in Table 3. IE and FA were more sensitive tests, when compared with serum and neutralization IDT. From our studies it is not clear whether IE was a more sensitive assay than FA. IE gave higher titers for serum samples 5, 6, and 7 but gave lower titers for samples 1, 2, 3, and 4 than those of FA.

DISCUSSION

This study showed that all three methods of PrV inactivation were effective and preserved antigenicity. However, there were differences in the quantities of antigenic determinants present

FIG. 8. IE quantitation of antigenic protein in BEI-treated antigen. (A) Standard PrV (0.21 mg), (B) nontreated control antigen (10 μ l), (C) BEI-treated (6 h) antigen (10 μ l).

FIG. 9. IE quantitation of antigenic protein in ^{60}Co -irradiated antigen. (A) Irradiated antigen (10 μ l), (B) nontreated control antigen (10 μ l), (C) standard PrV (0.21 mg).

after inactivation by the three techniques. Cobalt irradiation preserved least antigen, and BEI treatment preserved essentially all of the antigen contained in the sample. Photodynamic treatment resulted in more antigen being present after inactivation than was present in the control viral suspension, as measured in the IE system.

The mechanism by which acridine dye prevents viral maturation is not well understood at present. However, it is known that irradiation of protein and DNA with visible light in the presence of acridine dye leads to DNA-protein cross-linking (6, 17). It is possible that this cross-linking of viral DNA-protein complexes may interfere with the viral maturation process and thus lead to biological inactivation.

The concentration of acridine dye most often used to inactivate virions had been from 1 to 5

$\mu\text{g/ml}$ for animal viruses (13). At this concentration of dye, complexes of acridine with nucleic acid were usually formed (1, 22), and the structure of nucleic acid was distorted. Therefore, synthesis of nucleic acid was blocked, and the replication of infectious virus was inhibited remarkably. On the other hand, a low dose of acridine (0.08 $\mu\text{g/ml}$) was reported to have an insignificant effect on viral DNA synthesis (8). Since replication of infectious virus did not occur at this concentration of dye, the suggestion was made that a virus maturation step was interfered with or that defective particles were formed by such photodynamic treatment.

The increase in the ability of acridine dye-treated preparations to react with anti-PrV swine serum (Fig. 10) could be either due to the formation of more antigenic polypeptides in the

FIG. 10. IE quantitation of antigenic protein in photodynamically inactivated antigen. (A) Non-treated control antigen (30 μ l), (B) photodynamically inactivated antigen (30 μ l), (C) standard PrV (0.63 mg).

pool of defective virions for reaction with anti-PrV antibodies or due to the retention of critical molecular sizes or protein charges of immature virions in comparison with those of mature intact virions which increase anodal electrophoretic mobility during immunoanalysis.

The ^{60}Co -treated virus invariably showed less antigen activity than did the untreated control. This presumably is due to random hits in the gamma flux on both proteins and nucleic acid. The effect of such ionizing events on receptive molecules varies considerably with structure and size. However, cleavage occurs at weak bond linkages so that such moieties as halogen groups or certain ring structures are freed. As a consequence, in the process of denaturation of the nucleic acid, some proteins with antigenic activity are also apparently agitated and disrupted.

The use of standard antigen is essential for IE.

TABLE 3. Comparison of diagnostic tests for PRV antibody in swine serum

Serum sample	Serum neutralization (reciprocal titer)	IE (reciprocal titer)	IDT ^a	FA (reciprocal titer)
1	≥ 512	512	+++	4,096
2	≥ 512	1,024	++	4,096
3	≥ 512	512	++	4,096
4	64	128	++	256
5	≥ 512	512	++	256
6	≥ 512	1,024	+	256
7	4	32	—	4
8	≥ 512	1,024	++	1,024
9	≥ 512	1,024	+	1,024
10	≥ 512	1,024	+	1,024

^a +++, Very strongly positive; ++, strongly positive; +, positive; —, negative.

This assay technique is increasingly replacing the radial immunoassay. It has slight advantages over the radial immunoassay technique in that a peak linear span is easier to measure accurately than is the area of a circle, and results are obtained in much less time. It also has some advantages over other diagnostic tests (Table 3). It is simpler than FA or serum neutralization. It takes less time to obtain results for IE than it does for serum neutralization or IDT. It is not necessary to maintain sterile conditions during the manipulations. Serum samples found to be cytotoxic in serum neutralization tests do not interfere with IE.

The opaque precipitate bands illustrated in Fig. 4, 5, and 6 were due to specific reactions between antigen and antibody, as normal negative swine serum gave no precipitation. The multiple bands may have been caused by a so-called unbalanced system, i.e., excess of either antigen or antibody; this may give rise to migrating or multiple precipitates due to alternating solubilization and reprecipitation.

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