CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1996, p. 167–174 Vol. 3, No. 2
1071-412X/96/$04.00+0

An Indirect Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using Baculovirus-Expressed Antigen for the Detection of Antibodies to Glycoprotein E of Pseudorabies Virus and Comparison of the Method with Blocking ELISAs

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Received 27 September 1995/Returned for modification 21 November 1995/Accepted 20 December 1995

Antibodies in porcine sera against glycoprotein E (gE) of pseudorabies virus (PRV) are usually measured in blocking enzyme-linked immunosorbent assays (ELISAs) with one or two murine monoclonal antibodies (MAbs) directed against gE. Our aim was to develop a confirmation assay which is based on another principle and which is able to detect antibodies directed against most potential binding sites on gE with high specificity. Therefore, we developed an indirect double-antibody sandwich assay (IDAS) using recombinant gE expressed by baculovirus (BacgE960). A fragment of the gE gene consisting of nucleotide positions +60 to +1020 of gE, coding for the major antigenic sites of gE but not the transmembrane region, was cloned behind the signal sequence of PRV gG and the p10 promoter in a baculovirus vector. Immunoblot analysis showed that the expressed protein reacted with MAbs directed against five of the six antigenic sites on gE. Although the conformation of some antigenic sites, notably antigenic sites E and C, was not identical to their natural conformation, the expressed protein bound gE-specific antibodies in porcine sera in Western blots (immunoblots) and ELISAs. For the IDAS, a coating MAb directed against the nonimmunodominant antigenic site A on gE was chosen. A major obstacle in binding ELISAs, such as the IDAS, appeared to be the high nonspecific binding activity observed in porcine sera. As a result, sera could be tested only in relatively high dilutions in the BacgE960 IDAS, in contrast to the testing of sera in blocking ELISAs. The sensitivity and specificity of the newly developed BacgE960 IDAS were evaluated and compared with those of five commercially available blocking ELISAs by using several sets of sera of known PRV disease history. The BacgE960 IDAS assay had a high diagnostic specificity and a moderate sensitivity. The five blocking ELISAs differed remarkably in sensitivity and specificity, thereby illustrating the need for standardization and confirmation. We conclude that the BacgE960 IDAS is a useful and specific additional (confirmatory) test for the detection of antibodies to gE.

Vaccination of pigs against pseudorabies virus (PRV) (synonyms, Aujeszky's disease virus and suid herpesvirus type 1) with marker vaccines that lack the nonessential glycoprotein E (gE) (previously called gI) enables the detection of wild-type (wt) PRV-infected pigs in vaccinated populations by using serologic assays that detect antibodies to gE (37). This principle is currently used in control and eradication campaigns worldwide. A critical factor in these campaigns is the reliability, in terms of sensitivity, specificity, reproducibility, and robustness, of the serotests for gE-specific antibodies in porcine sera. Current serotests for gE-antibodies are blocking enzyme-linked immunosorbent assays (ELISAs) in which one or two monoclonal antibodies (MAbs) directed against gE are used. Reported difficulties in the detection of antibodies to gE include false-negative results, false-positive results, nonspecific reactions, high rates of doubtful and weakly positive test results, high interassay variability between batches of commercial test kits, low and variable gE-specific antibody responses in pigs with maternal antibodies, and the so-called single reactors (2, 31, 34, 36). A single reactor is a single gE-seropositive pig in a herd and may indeed reflect the only seropositive pig in a herd, i.e., a recently infected animal which has not or not yet resulted in transmission of the infection, or a false-positive test result (2). In addition, the success of vaccination-eradication campaigns results in a very low seroprevalence, which may be accompanied by a high rate of false-positive test results. To confirm with high predictive value such positive results, it is desirable to have a specific confirmation assay.

With murine MAbs, six antigenic domains, A to F, on gE have so far been identified (13). Sera of PRV-infected pigs predominantly recognize the conformational antigenic domain E and to a somewhat lesser degree recognize the antigenic domains C and D. Only a minority of infected pigs develop antibodies directed to the antigenic domains A, B, and F (12). The presently available gE serotests are blocking ELISAs which measure the inhibition of binding of murine MAbs directed against domain E or C or both by porcine antibodies directed to these antigenic domains. The purpose of the present study was to develop an additional, confirmation assay for the detection of antibodies to gE which is able (i) to detect antibodies in an assay which makes use of a principle other than the available blocking ELISAs used for screening, (ii) to detect gE-specific antibodies with high specificity without losing much sensitivity, and (iii) to detect antibodies directed against as many antigenic sites on gE as possible. This would also enable the serologic diagnosis of infections with potential

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aberrant PRV strains that have mutations in the antigenic domain E or C of gE (16). For that purpose, we expressed part of the gE gene, containing all antigenic sites identified so far, in the baculovirus system and developed an indirect double-antibody sandwich assay (IDAS) using the baculovirus-expressed gE as antigen (BacgE960 IDAS).

MATERIALS AND METHODS

MAbs. MAb 23.3.1a is directed against porcine immunoglobulin G (IgG) (38). Eleven MAbs directed against PRV gE have been described previously (13). MAbs 1, 3, and 5 are directed against the discontinuous but almost linear antigenic domain A (amino acids [aa] 78 to 239); MAbs 4, 8, and 11 are directed against the continuous antigenic domain B (aa 52 to 67); MAbs 6 and 9 are directed against the discontinuous antigenic domain C (aa 78 to 239); MAb 7 is directed against the continuous antigenic domain D (aa 68 to 82); MAb 2 is directed against the discontinuous antigenic domain E (aa 78 to 239); and MAb 10 is directed against the discontinuous antigenic domain F (amino acid range unknown). Antigenic domain E and, to a lesser extent, antigenic domains C and D are immunodominant in pigs. Only few pigs respond to antigenic domains A, B, and F (12). The MAbs were purified from mouse ascites fluid by 50% ammonium sulfate precipitation, which was followed by dialysis overnight against phosphate-buffered saline (PBS; pH 7.4).

Cells and viruses. The *Autographa californica* nuclear polyhedrosis virus (AcNPV) was used as vector for the expression of gE under the control of the p10 promoter. The methods have been described previously (9, 41). Briefly, AcNPV was grown in monolayers of the *Spodoptera frugiperda* cell line Sf21 (ATCC CRL 1711) (39) at 28°C in TC100 medium (GIBCO-BRL) (7) containing 10% heat-inactivated fetal bovine serum (Sebak 31054) and antibiotics (100 IU of penicillin per ml, 110 μg of streptomycin per ml, and 2.5 μg of fungizone per ml). For cotransfection, Sf21 cells were grown in Grace's insect tissue culture medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. For production of ELISA antigen, Sf21 cells were grown in SF900 serum-free insect culture medium (GIBCO-BRL 10900-066) supplemented with antibiotics. Virus titers were determined by end point dilution as described elsewhere (32).

Construction and characterization of recombinant baculovirus expressing gE. For the construction of recombinant baculovirus, the DNA of PRV strain NIA-3 (22) was digested with *Bam*HI. The *Bam*HI 7 fragment was subsequently cloned in vector pBR322 (Boehringer Mannheim), generating pBR322-Bam7. Plasmid DNA was recovered by standard methods (29). A fragment of 1,914 bp containing 1,020 bp of the gE gene was excised from vector pBR322-Bam7 by using *Apa*LI. The ends of the fragment were filled in with Klenow, and the fragment was subsequently cloned into the *Hin*cII site of pUC19 (Biolabs) (pUC19-ApaLIfr.). A fragment of 1,264 bp containing the first 370 bp of the gE gene was removed from pUC19-ApaIfr by using *Bam*HI and *Nco*I. This fragment was replaced by a 310-bp fragment consisting of nucleotide positions +60 to +370 of gE, which was obtained in a PCR with *Taq* polymerase (Perkin-Elmer). A *Bam*HI restriction site was introduced at the +60 position during PCR amplification. The resulting plasmid (pUC19-gE) contains the gE fragment without the gE signal sequence and without the C-terminal transmembrane region and was used to realize an in-frame connection with the signal sequence of the PRV envelope protein G (gG) in plasmid pARK6gGss. The pARK6gGss vector was obtained by cloning the gG signal sequence from plasmid pAcAs3gX into pARK6 (9). The gE-ApaLI fragment was excised from pUC19-gE by using *Bam*HI and *Hin*dIII and was cloned into pARK6gGss digested with *Bam*HI and *Xba*I. The sticky ends of *Hin*dIII and *Xba*I were filled in with Klenow before digestion with *Bam*HI. The resulting vector, pARKgE960, was used for cotransfection with AcNPV wt DNA.

For cotransfection, confluent monolayers of Sf21 cells (2×10^6) grown in T25 flasks were cotransfected in a 49-cm^2 petri dish (Falcon) with 2.5 μg of plasmid pARKgE960 DNA and 0.5 μg of baculovirus AcNPV wt DNA by the calcium phosphate coprecipitation method (32). Recombinant viruses expressing β-galactosidase were purified by six rounds of plaque purification under agarose with 2.5 μg of Bluo-Gal (GIBCO-BRL) per ml in a TC100 agar overlay as the indicator. Expression of gE was examined by Western blotting (immunoblotting) (see below). One recombinant expressing gE was used to prepare high-titer virus stocks ($>10^8$ 50% tissue culture infective doses [$TCID_{50}$] per ml) for further experiments and was designated BacgE960.

The optimal time and localization of expression of gE in cells and the supernatant of BacgE960-infected Sf21 cells were determined by infecting Sf21 cells at a multiplicity of infection of 10 $TCID_{50}$ per cell. Cells and cellular fractions were harvested each day until day 6 postinfection and were analyzed by Western blotting and ELISA for the expression of gE. Fractionation of cells was performed according to the method described by Summers and Smith (32). Briefly, the cells were harvested, centrifuged, and washed with PBS. The cells were suspended in lysis buffer (0.03 M Tris-HCl [pH 7.5], 0.01 M Mg acetate, 1% Nonidet P-40) and were incubated on ice for 10 min with occasional stirring. Then, the nuclei and membrane fractions were spinned down for 5 min at 2,000 × *g*, and the supernatant containing the cytoplasmic fraction was transferred to a new tube. The pellet was washed and resuspended in PBS.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described by Sambrook et al. (29). Briefly, proteins were precipitated with 10% TCA for 15 min. The pellets were washed twice in cold acetone and resuspended in sample buffer (10 mm Tris-HCl [pH 8.0], 1 mM EDTA, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.25% bromphenol blue). Resuspended proteins were denatured by being boiled for 5 min in sample buffer and were then subjected to electrophoresis in an SDS-12% PAGE gel using Laemmli's (19) discontinuous system in a Mini-Protean II Electrophoresis Cell (Bio-Rad). Separated proteins were electrophoretically transferred to a 45-μm-pore-size Protran cellulose nitrate membrane (Schleicher and Schuell) with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were washed twice in PBS containing 0.5 M NaCl, 5% horse serum (Sera-Tech 9904419), and 0.05% (vol/vol) Tween 80 (PBS-NHT buffer). The membranes were subsequently incubated for 1 h at room temperature with an optimal dilution of MAbs directed against gE. The membranes were then washed three times for 5 min in PBS-NHT buffer and were incubated with rabbit anti-mouse IgG-horseradish peroxidase conjugate (DAKO A/S; article no. P260) for 1 h at room temperature. The membranes were washed three times for 5 min in PBS-gT buffer and once in PBS and were finally incubated in substrate solution (0.5 mg of 3′-3′-diaminobenzidine, 0.001% H_2O_2).

Tunicamycin treatment. The cells were infected with BacgE960 at a multiplicity of infection of 10 and were incubated in serum-free medium containing 4 μg of tunicamycin (Sigma) per ml (14). Seventy-two hours postinfection, the cells and medium were harvested and analyzed by SDS-PAGE and immunoblotting.

Production of ELISA antigen. For production of ELISA antigen, approximately 2×10^7 Sf21 cells were infected with BacgE960 or, as a control, wt baculovirus (wt AcNPV) in a T175 flask at a multiplicity of infection of 2 to 5 $TCID_{50}$ per cell for 1.5 h at room temperature. The virus was removed, and the cells were supplied with 20 ml of SF900 serum-free insect culture medium and were grown at 28°C until all cells contained visible polyhedrins, which was usually at day 5 postinfection. The medium was collected, clarified by centrifugation for 10 min at 600 × *g*, and subsequently filtered through a 0.2-μm-pore-size filter. The antigen was stored at −20°C before use in the IDAS.

IDAS. The test was designed to detect the binding of porcine IgG antibodies to recombinant gE (BacgE960) bound to the wells of a microtiter plate by a catching MAb directed to a linear nonimmunodominant epitope on gE (MAb 5). All reagents were added in 100-μl quantities. After each incubation step, the plates were washed six times with a solution of 0.05% Tween 80 in tap water. For coating, ELISA plates (Costar E.I.A. 3590) were incubated overnight at 37°C with MAb 5 dissolved in PBS. The plates were subsequently incubated for 1 h at 37°C with an optimal dilution of the baculovirus-expressed gE antigen (clarified and filtered culture supernatant of BacgE960-infected Sf21 cells). A control plate was similarly incubated with supernatant of wt AcNPV-infected Sf21 cells. Serum test samples, diluted 1:20 in test buffer consisting of PBS containing 20% horse serum (Sera-Tech 9904419), 0.5 M NaCl, and 2% Tween 80, were then pipetted into the wells of the antigen-containing and control plate and incubated for 30 min at 37°C. To determine ELISA titers, serial twofold dilutions of the test samples were made in the test buffer. An optimal dilution of the conjugate was prepared in PBS containing 4% fetal bovine serum, and the dilution was incubated for 60 min at 37°C. Conjugate was prepared by coupling the anti-porcine IgG MAb 23.3.1a to horseradish peroxidase (Boehringer Mannheim; article no. 814407) according to the method described by Wilson and Nakane (44). The conjugate was dialyzed against PBS, after which glycerol was added to a final concentration of 50%. The conjugate was stored at −20°C and, immediately before use, was diluted in PBS containing 4% fetal bovine serum. Finally, 100 μl of the substrate-chromogen mixture, consisting of 3,3′,5,5-tetramethylbenzidine (Sigma; 1 mg/ml) and H_2O_2 (0.005%) in 0.1 M Na-acetate buffer (pH 6.0), was added. After incubation for 15 min at room temperature, the reaction was stopped by the addition of 100 μl of 0.5 M H_2SO_4. Color development was measured at 450 nm with a microplate reader (EAR 400; SLT-Labinstruments, Vienna, Austria).

The following controls were included in each assay. As mentioned, every test was done in a plate with gE antigen, yielding an optical density at 450 nm for gE [OD_{450}(gE)], and in a plate with control antigen, yielding an OD_{450}(control). Four wells in row 12 of each plate contained twofold dilutions of a positive reference serum sample, two wells were filled with a negative serum sample, and two wells were filled with buffer during the serum incubation step. A sample (or sample dilution) was scored as positive when the OD_{450}(gE) minus OD_{450} (control) was >0.4. The test was considered valid when (i) the positive control serum sample scored positive in all four dilutions, (ii) the negative control serum sample scored negative in both wells, and (iii) the control wells tested without serum showed OD_{450} of ≤0.04.

Validation of the BacgE960 IDAS and comparison with blocking ELISAs. The newly developed BacgE960 IDAS was validated with 395 well-defined sera and was compared with five commercially available blocking ELISAs for the detection of antibodies to PRV gE (Svanova, Uppsala, Sweden; Rhone Merieux, Lyon, France; Eurodiagnostica, Apeldoorn, The Netherlands; Idexx, Portland, Maine, European Veterinary Laboratories, Woerden, The Netherlands). The five commercially available blocking ELISAs were designated (in random order)

from A to E. The samples were analyzed in the blocking ELISAs strictly according to the instructions of the manufacturers. Tests B and E are based on the principle of the complex trapping blocking ELISA (8), which makes use of a preincubation step of test serum and antigen in a separate plate; transfer of the serum-antigen mixture to the test plate, which is coated with one anti-gE MAb; and a conjugate step with a second anti-gE MAb. Hence, these tests detect porcine antibodies against one or two antigenic sites on gE. Tests A, C, and D are based on the principle of a direct blocking ELISA, which makes use of an antigen-coated plate, a serum incubation step, and a conjugate step with an anti-gE MAb. Hence, these tests detect porcine antibodies against one antigenic site on gE. In ELISAs B, C, and E, undiluted serum is used, whereas in ELISAs A and D, serum samples must be diluted 1:2.

The following serum collections were available for this purpose (i). Ten weakly positive and 17 negative serum samples were obtained from specific-pathogen-free (SPF) pigs from our institute and several other European laboratories. The pigs had been vaccinated and infected according to various protocols to induce low to very low levels of antibodies to gE or to give high levels of antibodies to PRV without antibodies to gE. Some of these serum samples have been selected by the Subcommittee on Aujeszky's disease of the European Union Scientific Veterinary Committee. (ii) Sixteen positive and 56 negative swine serum samples were obtained from pigs with a known history of PRV. Positive serum samples were obtained from pigs that were infected with wt PRV or that were vaccinated with gE-positive vaccines. Negative samples were obtained from PRV-free herds, either vaccinated with gE-negative vaccines or not vaccinated, in the United Kingdom and the Netherlands. (iii) An additional set of 249 negative samples was obtained from PRV-free herds from the United Kingdom. This set was used only to examine the specificity of the BacgE960 IDAS. (iv) To determine the detection limit of each test, serial twofold dilutions (1:2 to 1:2,048 for the blocking ELISAs and 1:40 to 1:40,960 for the BacgE960 IDAS) of 14 positive serum samples were made in negative SPF serum. (v) In addition, a collection of six well characterized serum samples were used to further analyze the detection limit of each method. These six samples were used in an interlaboratory exchange program according to ISO-5725 (10) to check the sensitivity, specificity, reproducibility, and repeatability of gE antibody tests in different laboratories in the Netherlands. (vi) The ability of each method to detect antibodies early after infection was evaluated by testing serum samples obtained from a pig that was vaccinated intranasally with the PRV Bartha strain and challenge inoculated intranasally with 10^5 PFU of the virulent NIA-3 strain (22) at 3 weeks postvaccination. Blood samples were collected at days 8, 11, 15, 18, 21, and 76 after challenge inoculation. (vii) To further determine the specificity of the tests, 21 serum samples of different origins were analyzed in each test: eight nonimmune calf serum samples, four bovine herpesvirus type 1 (BHV1)-immune calf serum samples, one equine arteritis virus-immune horse serum sample, one porcine parvovirus-immune pig serum sample, one porcine influenza virus H3N2-immune pig serum sample, and one porcine influenza virus H1N1-immune pig serum sample, and five *Streptococcus suis* (type 2)-immune pig serum samples were available for this purpose. With the exception of the 249 negative serum samples from the United Kingdom, all test samples were analyzed by each of the six methods. On the basis of the results obtained with the negative and positive serum samples, specificity and sensitivity were determined, respectively. The specificity of an ELISA was expressed as the percentage of the negative serum samples obtained from animals never infected with PRV which gave unambiguously negative results. According to a worst-case scenario, we considered doubtful results positive. The sensitivity of an ELISA was expressed as the percentage of the positive serum samples obtained from animals (experimentally) infected with PRV which gave unambiguously positive results. Doubtful results were considered negative.

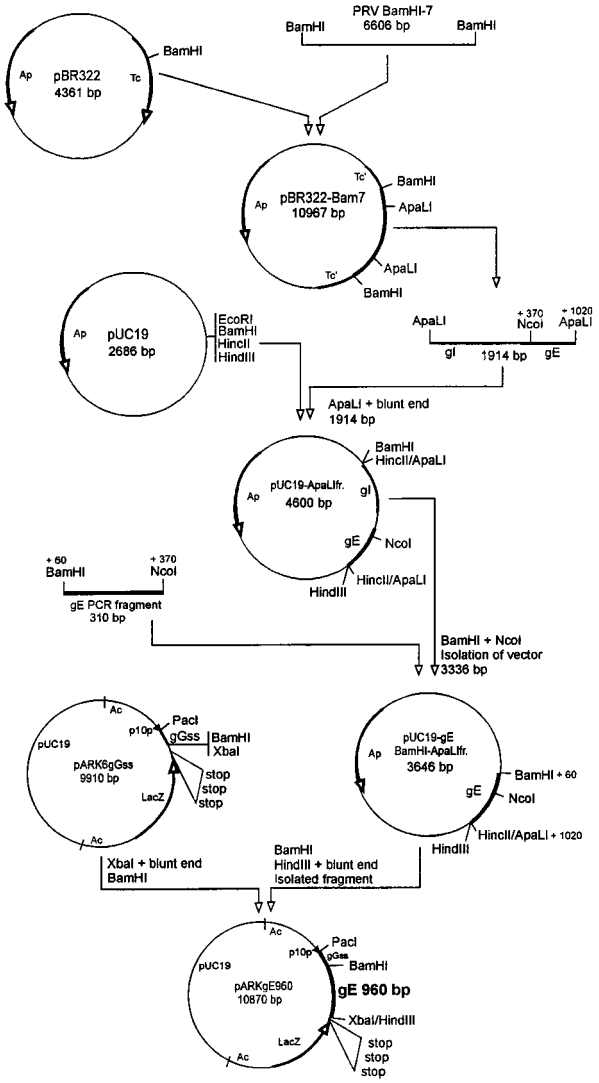

FIG. 1. Construction of transfer vector pARKgE960. Fragments of PRV gE were cloned into the multicopy vector pUC19 and then subcloned into the transfer vector pARK6. Arrows indicate the directions of transcription. Ac, AcNPV DNA; Ap, ampicillin resistance gene; Tc, tetracycline resistance gene; p10p, p10 promoter; gGss, PRV gG signal sequence; LacZ, *Escherichia coli lacZ* gene; STOP STOP STOP, stop codons in three reading frames.

RESULTS

Expression of PRV gE by baculovirus. PRV gE was cloned behind the N-terminal signal sequence of PRV gG to allow efficient intracellular transport. The C-terminal transmembrane anchor was removed from the construct to allow efficient secretion into the supernatant. The construction of the pARKgE 960 transfer vector is schematically shown in Fig. 1. One recombinant baculovirus, which was designated BacgE960, was used to prepare high-titer virus stocks, to characterize the expressed gE product, and to develop the IDAS for the detection of porcine antibodies to gE.

The localization of the recombinant BacgE960 product was checked by Western blot analysis of the supernatant of BacgE 960-infected Sf21 cells, as well as of nuclear, membrane, and cytoplasmic fractions. BacgE960 was demonstrated in all fractions. The cytoplasmic fraction contained a mixture of heterogeneously glycosylated proteins with molecular masses of 42 to 46 kDa (data not shown), which is in accordance with the molecular mass predicted from the DNA sequence (about 44 to 46 kDa). The nuclear and membrane fractions contained a gE protein with a molecular mass of approximately 42 kDa, suggesting that this form of gE is poorly glycosylated. In the supernatant, gE was mainly expressed as a doublet with molecular masses of 44 and 46 kDa. Tunicamycin treatment of BacgE960-infected Sf21 cells and then Western blot analysis resulted in one band with an apparent molecular mass of 38 kDa, therewith revealing heterogeneous N-linked glycosylation of the expressed gE polypeptides of 42 to 46 kDa (data not shown). The heterogeneity of the glycoprotein bands from 42 to 46 kDa is consistent with the presence of four potential glycosylation sites as determined by computer sequence analysis. Tunicamycin treatment reduced the amount of secreted gE, while the 38-kDa form accumulated intracellularly. These results confirm those of Jarvis and Summers (14), who showed that glycosylation is a prerequisite for efficient secretion of a protein in baculovirus-infected insect cells.

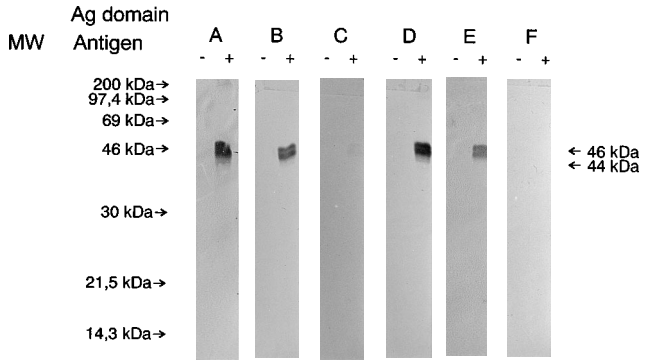

FIG. 2. Antigenicity of BacgE960 examined by gel electrophoresis and Western blot analyses with BacgE960 antigen (Ag) (+) and control antigen (−) (supernatant of wt AcNPV-infected cells). Lane samples were incubated with MAb 5 (representative of antigenic domain A), MAb 8 (representative of antigenic domain B), MAb 9 (representative of antigenic domain C), MAb 7 (representative of antigenic domain D), MAb 2 (representative of antigenic domain E), and MAb 10 (representative of antigenic domain F). Molecular mass calibrations are indicated to the left of the blot.

When cells were infected at a multiplicity of infection of 10 $TCID_{50}$ per cell, the expression reached a maximum level at day 3 postinfection before the cells began to lyse.

Antigenicity of BacgE960. To determine whether the baculovirus-expressed antigen contained all identified antigenic domains of gE, Western blot analyses were performed with murine MAbs representative for each of the antigenic domains A to F (Fig. 2). With the exception of MAb 10, directed against the discontinuous antigenic domain F, the MAbs recognized the 44- and 46-kDa protein doublet and sometimes a faint and diffuse 42-kDa band as well. However, MAb 2 and MAbs 6 and 9, respectively, directed against the discontinuous antigenic domains E and C (12), reacted with faint or intermediate bands. These positive results were somewhat surprising, because we have previously shown that the conformation of the discontinuous antigenic domains E and C (but not of the linear antigenic domains A and D) is partly dependent on the noncovalently linked complex between gE and gI (11). However, BacgE960 reacted poorly (in comparison with wt NIA-3 antigen) in an ELISA with MAbs 2 and 9 as catching and detecting antibodies, respectively. We concluded that the BacgE960 antigen contained most of the major identified antigenic domains on gE. However, the conformation of the antigenic domains E and C is most likely not completely identical to its natural conformation.

Development of the BacgE960 IDAS. The ELISA with the BacgE960 as antigen was set up by using 30 negative and 20 positive serum samples with known history as reference material. Incubation temperatures and periods, type and pH of buffers, washing conditions, coating material, serum dilutions, blocking solutions, concentrations of reagents, and cutoff values were chosen in a series of checkerboard titrations. Because the purpose of the work was to develop an assay which can be used to confirm the results of screening ELISAs in the latter stages of eradication-control programs (in which the prevalence of the disease may be very low), the assay was set up to have a high specificity with an acceptable level of sensitivity. It further appeared necessary to diminish the nonspecific binding of the porcine serum samples to the ELISA plate as much as possible. Sera from conventional pigs from the field gave higher nonspecific binding than sera from SPF pigs.

During the development of the assay, it appeared that the sensitivity could be improved by coating a MAb directed against PRV to catch the antigen to the wells of the microtiter plate instead of coating the antigen directly. Because the use of a coating MAb could block the binding to the antigen of antibodies in test samples (and thus reduce the sensitivity of the test), we selected MAb 5, which is directed against antigenic domain A on gE and to which only a small proportion of PRV-infected pigs develop antibodies (12). The final setup of the assay was described in Materials and Methods and is shown schematically in Fig. 3.

To test the reproducibility of the BacgE960 IDAS, two strongly positive and two weakly positive serum samples were tested 7 times on different days over a 3-month period. The mean $[OD_{450}(gE) - OD_{450}(control)] \pm$ standard deviation values obtained with these serum samples were 1.82 ± 0.10, 1.56 ± 0.09, 1.03 ± 0.16, and 0.62 ± 0.09, yielding low and acceptable coefficients of variation of 5.5, 5.8, 15.6, and 14.6%, respectively.

Attempts were further made to develop a Western blot assay with BacgE960 as the antigen for the detection of antibodies directed against gE in porcine sera. These attempts were not pursued because of the lower degree of sensitivity of Western blot analysis in comparison with the IDAS.

Validation of the BacgE960 IDAS and comparison with blocking ELISAs. The reliability of the newly developed BacgE 960 IDAS was determined and simultaneously compared with those of five commercially available blocking ELISAs (A to E) by using several sets of well-defined sera. All samples were tested according to the protocol of the BacgE960 IDAS or the instructions of the manufacturer. However, tests A and E did not fulfill the criteria of validity as given by the manufacturer, i.e., these tests failed to reach the prescribed results with control sera.

First, the tests were evaluated by testing 10 weakly positive and 17 negative serum samples from SPF pigs which had been vaccinated and infected in different laboratories. The pigs were treated according to protocols to give low to very low levels of antibodies to gE or to give high levels of antibodies to PRV without antibodies to gE. As shown in Table 1 (first row), the BacgE960 IDAS failed to detect these low levels of gE antibodies in 5 of 10 samples but showed a high specificity. Tests A to E showed various results. Second, the tests were evaluated by using 16 positive and 56 negative serum samples from pigs with a well-known PRV history (Table 1, second row). The combined data obtained with all positive (10 plus 16) and negative (17 plus 56) serum samples are given in Table 1, third row. These data show that the BacgE960 IDAS combines a high level of specificity (99%) with a slightly lower level of

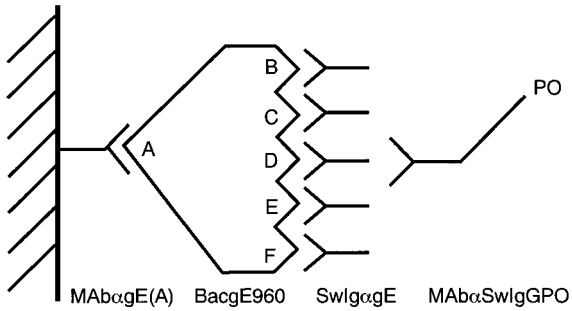

FIG. 3. Schematic representation of the BacgE960 IDAS. MAbαgE(A), coating MAb directed against antigenic domain A of gE; BacgE960, baculovirus-expressed gE antigen (BacgE960) containing antigenic sites A to E; SwIgαgE, test serum containing antibodies directed against gE; MAbαSwIgGPO; peroxidase (PO)-labelled MAb directed against swine IgG.

TABLE 1. Results obtained by BacgE960 IDAS and ELISAs A to E with known positive and negative serum samples

Assay	Result (%) for					
	Sera from experimentally inoculated SPF animals (n = 27)		Sera from pigs with a known PRV history (n = 72)		Totals (n = 99)	
	Specificity[a]	Sensitivity[b]	Specificity[a]	Sensitivity[b]	Specificity[a]	Sensitivity[b]
BacgE960 IDAS	100 (17/17)	50 (5/10)	98 (55/56)	100 (16/16)	99 (72/73)	81 (21/26)
Test A	100 (17/17)	80 (8/10)	95 (53/56)	94 (15/16)	96 (70/73)	88 (23/26)
Test B	100 (17/17)	90 (9/10)	96 (54/56)	100 (16/16)	97 (71/73)	96 (25/26)
Test C	88 (15/17)	100 (10/10)	84 (47/56)	100 (16/16)	85 (62/73)	100 (26/26)
Test D	100 (17/17)	90 (9/10)	100 (56/56)	100 (16/16)	100 (73/73)	96 (25/26)
Test E	100 (17/17)	70 (7/10)	91 (51/56)	100 (16/16)	93 (68/73)	88 (23/26)

[a] Numbers in parentheses are the numbers of negative test results/numbers of tested negative sera.
[b] Numbers in parentheses are the numbers of positive test results/numbers of tested positive sera.

sensitivity (81%). Remarkably, the commercially available ELISAs A to E differed strongly in sensitivity and specificity. Tests B and D combined high levels of sensitivity and specificity, with test D being slightly more superior in specificity. In addition to not fulfilling the criteria of validity given by the manufacturer, tests A and E showed a relatively low level of sensitivity and a moderate level of specificity. Test C revealed the highest sensitivity of the tests. However, in contrast to the high level of sensitivity was its relatively low specificity of 85%. In a number of cases, negative samples were scored as doubtful by test C. Third, the specificity of the BacgE960 IDAS was further examined by testing 249 serum samples from PRV-free herds from the United Kingdom. Only three of these samples reacted positively, giving a specificity of 99%. Fourth, to compare the detection level of each test, 14 selected positive serum samples were serially diluted twofold in negative SPF swine serum and analyzed. The highest dilution of each serum sample giving a positive result was calculated (Table 2). These data indicate that on average the BacgE960 IDAS gave the highest dilution giving a positive sample result. Fifth, to further determine the detection limit of each method, six well-characterized serum samples were analyzed in each test. These six samples are used in an interlaboratory exchange program (10) to check the sensitivity, specificity, reproducibility, and repeatability of gE antibody tests in different laboratories. On the basis of the results obtained over a 2-year period in the participating laboratories, the sera had arbitrarily been designated as sera containing no, very low, low, medium, high, or very high levels of gE antibodies. As shown in Table 3, the BacgE960 IDAS detects gE antibodies in the samples containing medium and higher levels of gE antibodies, but not in the samples containing no, very low, or low levels of gE antibodies. This apparent contradiction between a high detection limit as determined by diluting positive sera and a somewhat lower diagnostic sensitivity when the test is used at a single dilution is due to the high sample dilution (i.e., 1:20) which had to be used in the BacgE960 IDAS to circumvent the nonspecific binding of porcine sera. Sixth, the ability of each method to detect gE antibodies shortly after infection was evaluated by analyses of serially collected serum samples obtained from a pig which was vaccinated and subsequently infected. With the exception of tests A (day 76) and C (day 11), all tests detected infection at day 15 postinfection. Seventh, to further determine the specificities of the tests, 21 serum samples of different origins were analyzed in each test. These serum samples gave negative scores in all but one test; test E gave doubtful scores for two BHV1-seronegative calf serum samples (data not shown).

TABLE 2. Sensitivities of the BacgE960 IDAS and ELISAs A to E as defined by the highest dilution giving a positive test result

Serum sample no.	Highest dilution giving a positive result for:					
	BacgE960IDAS	Test A	Test B	Test C	Test D	Test E
1	≤20	16	8	16	8	2
2	320	64[a]	16	64	16	4
3	≤20	32	16	64	16	8
4	≤20	16[a]	8[a]	64	64[a]	4
5	128	256	128	128	64	32
6	2,560	1,024	1,024	1,024	1,024	128
7	640	32	16	32	32	4
8	40	24	48	24	48	2[a]
9	≤20	16	2	32	≤1[a]	≤1
10	640	64	32	128	32	16
11	1,280	64	64	256	64	16
12	2,560	≥2,048	≥2,048	≥2,048	≥2,048	512
13	≤20	2	≤1	4	≤1	≤1[a]
14	10,240	512[a]	128	512	128	64
Mean[b]	200	63	32	79	40	10

[a] Serial dilutions gave inconclusive results.
[b] Geometric means. To allow calculation, negative values were set at the detection level and values greater than or equal to and less than or equal to were read as equal to the respective values.

DISCUSSION

We report the synthesis of a truncated gE antigen from a recombinant virus expression system for application in a diagnostic test. Several previous attempts in our laboratory to develop recombinant gE for use in an immunodiagnostic assay were unsuccessful, either because of inappropriate processing and conformation of recombinant gE in bacterial cells, a low level of expression of recombinant gE in eukaryotic 3T3 cells, or unsuccessful expression of full-length gE in bacterial and

TABLE 3. Sensitivities and specificities of the BacgE960IDAS and ELISA A to E as defined by six test serum samples

Serum sample (antibody level)[a]	Result with:					
	BacgE960IDAS	Test A	Test B	Test C	Test D	Test E
1 (negative)	−	−	−	−	−	−
2 (very low)	−	±	−	±	−	+
3 (low)	−	+	+	+	+	−
4 (medium)	+	+	+	+	+	+
5 (high)	+	+	+	+	+	+
6 (very high)	+	+	+	+	+	+

[a] Level of gE-specific antibodies.

insect cells (results not shown). The last finding may be due to the toxicity of mature full-length gE, as suggested by the instability of recombinant baculovirus-expressing full-length gE (results not shown). A possible explanation for toxicity may be that accumulation of full-length gE, including the transmembrane region, in the membranes of the endoplasmic reticulum may inhibit protein syntheses, resulting in low levels of production of the expressed protein (9). Therefore, we decided to express a truncated part of gE containing the major antigenic part of gE in the baculovirus expression system (13). Because most aspects of the intracellular modification of proteins in insect cells are similar to those in eukaryotic cells (1, 18, 21, 25), conformation-dependent antigenic sites of baculovirus-expressed proteins may largely have the native structure, therewith facilitating their use in immunodiagnostic assays, as previously shown (4, 27, 33, 35, 40, 42). A correct folding of the expressed gE is important, because we previously showed that the major immunodominant antigenic sites on gE are conformation dependent (12, 13). In addition, the level of expression of heterologous proteins in the baculovirus-insect cell system is high (18, 21, 25). The PRV gG signal sequence was introduced to allow efficient intracellular transport. Because the expressed BacgE960 contained no transmembrane region, the expressed protein was excreted, although partly, in the supernatant of BacgE960-infected insect cells, allowing an easy harvest of recombinant proteins. Because it has been previously shown that secretory and membrane proteins are excreted properly only if they are correctly folded and oligomerized in the endoplasmic reticulum (9, 20, 26), excretion of the BacgE960 product in the supernatant of infected cells is a further indication of a correct processing and folding of the recombinant antigen. Western blotting (Fig. 2) indicated that the expressed BacgE960 antigen contained most of the identified antigenic sites, including the immunodominant conformational antigenic domains C and E.

Although BacgE960 was clearly antigenic in the Western blot analysis, two lines of evidence suggested that the antigenicity of the expressed BacgE960, especially of the immunodominant conformational antigenic domains, is not identical to that of gE in wt PRV virions or virus-infected cells. First, BacgE960 reacted poorly (in comparison with complete viral antigen) in a blocking ELISA with MAbs to antigenic sites E and C as catching and detecting antibodies. Second, we have previously shown that the binding of MAbs directed to antigenic domains E and C was enhanced by complexing gE to gI (previously called gp63) (11). In PRV virions and in PRV-infected cells, gE forms a noncovalently linked complex with gI (43, 46). Not only in PRV, but also in herpes simplex virus type 1 (15), BHV1 (28), varicella-zoster virus (45), and Marek's disease virus (3), gE and gI homologous proteins are noncovalently bound. In PRV, the gE-gI complex (previously called gI-gp63 complex) is a functional and apparently also antigenic entity (6, 11, 43, 46). These findings indicate that application of a gE-gI complex as antigen for the detection of gE-specific antibodies may have advantages. This is possible in blocking ELISAs but impossible in binding assays such as the IDAS; note that vaccinated pigs may develop gI-specific antibodies. Nonetheless, despite the intrinsic shortcomings of the expressed BacgE960, it appeared sufficiently antigenic for use in ELISAs and Western blot assays. The developed BacgE960 IDAS detects gE-specific porcine IgG. In an antibody capture assay, the antigen would also be able to detect gE-specific IgM (17).

An advantage of an IDAS with recombinant or purified (23) gE is its potential ability to detect antibodies directed against several antigenic determinants on the protein, in contrast to that of the current commercially available blocking ELISAs, which detect antibodies directed against only one or two epitopes. Therefore, the BacgE960 IDAS may have the ability to detect infection with PRV strains that have mutations in antigenically important regions of gE. The occurrence of such strains may impair PRV eradication campaigns in which the blocking ELISAs are used as a diagnostic test to discriminate infected from vaccinated pigs. One such strain has been described by Katz and Pederson (16). In addition, a partially gE-deleted PRV isolate from the field with a low level of expression of a truncated form of gE has been described elsewhere (24). The importance of gE serotests for PRV eradication campaigns illustrates the necessity of both the characterization of the gE phenotype of new isolates and of serological surveillance by the BacgE960 IDAS.

Because the prevalence of gE-seropositive pigs in vaccinated herds may be very low (5, 31), a sensitive test and large sample size are needed to detect positive herds. However, because high sensitivity usually combines with low specificity (see, for example, test C), this will likely result in a large number of false-positive test results. We developed the BacgE960 IDAS to have a high specificity so that it can be used to confirm such positive test results in herds with a low prevalence, thereby enhancing the predictive value of positive test results.

For the evaluation of the diagnostic quality of the BacgE960 IDAS and the commercial tests A to E, we used several sets of well-characterized sera from pigs with known PRV histories regarding both natural and experimental infections. The advantage of this approach is that sensitivity and specificity can be calculated unambiguously, which is difficult or impossible when sera from the target population itself are used, because the infection history is usually unknown. However, the choice of sera may bias the results. In addition, some sera from experimental infections may not be representative for the field situation (30). For example, the first set of sera (Table 1, first row) contained experimental sera with low to very low concentrations of gE antibodies. Because the BacgE960 IDAS was developed to have a high specificity, it conversely had a relatively lower sensitivity, which was especially clear with this collection of sera. The second set of sera (Table 1, second row) may be more representative for the field situation. With this set of sera, the BacgE960 IDAS gave a higher-sensitivity score in addition to its high specificity. Other sets of sera (including sera from PRV-free herds from the United Kingdom) were further included to test the specificity of the BacgE960 IDAS. Because PRV gE shows sequence homology with gEs of other alphaherpesviruses, we also examined the possibility that the BacgE960 IDAS detected anti-BHV1 gE-specific antibodies in calf sera cross-reacting with PRV gE. However, the four BHV1-immune calf serum samples all reacted negatively in the BacgE960 IDAS. We concluded from the validation of tests that several commercially available ELISAs differed remarkably in quality (test B and especially test D had high levels of sensitivity and specificity), and that the BacgE960 IDAS had a high level of specificity and an acceptable level of sensitivity. The different degrees of reliability of the commercial tests and their high interassay variability (34) point to the need for rigid standardization as well as for use of a confirmatory test. When used to confirm positive results of screening assays, the BacgE960 IDAS will increase the predictive value of ultimately positive test results. The BacgE960 IDAS may further be useful as an additional confirmation assay because some commercial assays (notably tests A, C, and E) detect only antibodies against a single epitope. Nonetheless, when the BacgE960 IDAS is used as a confirmation assay, it should be kept in mind that the test may fail to detect a sample with a low level of gE

antibodies. This will not have serious implications for eradication of PRV, provided that the swine population is thoroughly vaccinated (31).

In conclusion, we have obtained a high level of expression of a truncated gE in the baculovirus expression system. The expressed BacgE960 was sufficiently antigenic to develop an IDAS for the detection of antibodies directed against gE in swine sera. The developed BacgE960 IDAS had a high level of specificity and an acceptable level of sensitivity, both of which make the test a useful complementary or confirmatory test for the diagnosis of wt PRV infections. The test may be further improved by circumventing the nonspecific binding of porcine sera. For that purpose, we are attempting to develop a recombinant gE antigen with a specific tag.

ACKNOWLEDGMENTS

We thank M. Banks (Central Veterinary Institute, Weybridge, United Kingdom), H. van der Heijden (Regional Animal Health Service Laboratory in the Southern Netherlands), and U. Vecht (IDDLO) for providing sera with known disease histories. M. Hulst and J. Quak were helpful in introducing the baculovirus expression system and providing technical assistance.

This work was supported by KBN grant 411619101 and a grant from the Animal Health Service.

REFERENCES

1. **Altmann, F., G. Kornfeld, T. Dalik, E. Staudacher, and J. Glossl.** 1993. Processing of asparagine-linked oligosaccharides in insect cells. *N*-Acetylglucosaminyltransferase I and II activities in cultured lepidopteran cells. Glycobiology **3:**619–625.
2. **Annelli, J. F., R. B. Morrison, S. M. Goyal, M. E. Bergeland, W. J. Mackey, and D. G. Thawley.** 1991. Pig herds having a single reactor to serum antibody tests to Aujeszky's disease. Vet. Rec. **128:**49–53.
3. **Brunovskis, P., X. Chen, and L. F. Velicer.** 1992. Analysis of Marek's disease glycoproteins D, I, and E, p. 118–121. Proceedings of the 19th World's Poultry Congress. Amsterdam, the Netherlands.
4. **Chuma, T., H. Le Blois, J. M. Sanchez-Vizcaino, M. Diaz-Laviada, and P. Roy.** 1992. Expression of the major core antigen VP7 of African horsesickness virus by a recombinant baculovirus and its use as a group-specific diagnostic reagent. J. Gen. Virol. **73:**925–931.
5. **Duffy, S. J., R. B. Morrison, and D. G. Thawley.** 1990. Spread of pseudorabies virus within quarantined swine breeding herds. JAMA **199:**61–65.
6. **Enquist, L. W., J. Dubin, M. E. Whealy, and J. P. Card.** 1994. Complementation analysis of pseudorabies virus gE and gI mutants in retinal ganglion neurotropism. J. Virol. **68:**5275–5279.
7. **Grace, T.** 1962. Establishment of four strains of cells from insect tissues grown *in vitro*. Nature (London) **195:**788–789.
8. **Houwers, D. J., and J. Schaake.** 1987. An improved ELISA for the detection of antibodies to maedi-visna and caprine arthritis-encephalitis virus, employing monoclonal antibodies in a novel one-step blocking concept. J. Immunol. Methods **98:**151–154.
9. **Hulst, M. M., D. F. Westra, G. Wensvoort, and R. J. M. Moormann.** 1993. Glycoprotein E1 of hog cholera virus expressed in insect cell protects swine from hog cholera. J. Virol. **67:**5435–5442.
10. **International Organization for Standardization.** 1986. International standard ISO 5725. Precision of test methods—determination of repeatability and reproducibility for a standard test method by inter-laboratory tests, 2nd ed. Publication no. UDC 519.248:620.1. International Organization for Standardization, Lausanne, Switzerland.
11. **Jacobs, L., O. de Leeuw, F. Hanssens, and T. G. Kimman.** 1995. Complex between glycoproteins gE and gI of pseudorabies virus is important for optimal presentation of conformation-dependent immunodominant epitopes on gE, p. 132–136. *In* M. Schwyzer and M. Ackermann (ed.), Immunobiology of viral infections. Proceedings of the 3rd Congress of the European Society of Viral Virology.
12. **Jacobs, L., and T. G. Kimman.** 1994. Epitope-specific antibody responses against glycoprotein E of pseudorabies virus. Clin. Diagn. Lab. Immunol. **1:** 500–505.
13. **Jacobs, L., R. H. Meloen, A. L. J. Gielkens, and J. T. van Oirschot.** 1990. Epitope analysis of glycoprotein I of pseudorabies virus. J. Gen. Virol. **71:** 881–887.
14. **Jarvis, D. L., and M. D. Summers.** 1989. Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. Mol. Cell. Biol. **9:**214–223.
15. **Johnson, D. C., and V. Feenstra.** 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. **61:**2208–2216.
16. **Katz, J. B., and J. C. Pederson.** 1992. Analysis of glycoprotein I (gI) negative and aberrant pseudorabies viral diagnostic isolates. Am. J. Vet. Res. **53:** 2259–2263.
17. **Kimman, T. G.** 1993. Characterization of the pseudorabies virus-specific immunoglobulin M response and evaluation of its diagnostic use in pigs with preexisting immunity to the virus. J. Clin. Microbiol. **31:**2309–2314.
18. **King, L., and R. Possee (ed.).** 1992. The baculovirus expression system: a laboratory guide. Chapman and Hall, London.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
20. **Lodisch, H. F.** 1988. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. J. Biol. Chem. **263:** 2107–2110.
21. **Luckow, V., and M. Summers.** 1988. Trends in development of baculovirus expression vectors. Bio/Technology **6:**47–55.
22. **McFerran, J. B., and C. Dow.** 1975. Studies on immunization of pigs with the Bartha strain of Aujeszky's disease virus. Res. Vet. Sci. **19:**17–22.
23. **Mellencamp, M. W., N. E. Pfeiffer, B. T. Suiter, J. R. Harness, and W. H. Beckenhauer.** 1989. Identification of pseudorabies virus-exposed swine with a gI glycoprotein enzyme-linked immunosorbent assay. J. Clin. Microbiol. **27:**2208–2213.
24. **Mettenleiter, T. C., C. Schreurs, H. J. Thiel, and H.-J. Rziha.** 1987. Variability of pseudorabies virus glycoprotein I expression. Virology **158:**141–146.
25. **Miller, L.** 1988. Baculovirus as gene expression vectors. Annu. Rev. Microbiol. **42:**177–199.
26. **Pfeffer, S., and J. Rothman.** 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu. Rev. Biochem. **56:**829–852.
27. **Reid-Sanden, F. L., J. W. Sumner, J. S. Smith, M. Fekadu, J. H. Shaddock, and W. J. Bellini.** 1990. Rabies diagnostic reagents prepared from a rabies N gene recombinant expressed in baculovirus. J. Clin. Microbiol. **28:**858–863.
28. **Rijsewijk, F. A. M.** Unpublished data.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Stegeman, A., M. C. M. de Jong, H. M. J. F. van der Heijden, A. R. W. Elbers, and T. G. Kimman.** Evaluation of tests for detection of antibodies to pseudorabies virus glycoprotein E in the target population. Unpublished data.
31. **Stegeman, A., T. G. Kimman, J. T. van Oirschot, M. M. J. M. Tielen, and W. A. Hunneman.** 1994. Spread of pseudorabies virus within pig herds in an intensively vaccinated region. Vet. Rec. **134:**327–330.
32. **Summers, M. D., and E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Bulletin no. 1555. Texas Agricultural Experimental Station, College Station, Tex.
33. **Urukawa, T., and P. Roy.** 1988. Bluetongue virus tubules made in insect cells by recombinant baculoviruses: expression of the NS1 gene of bluetongue virus serotype 10. J. Virol. **62:**3919–3927.
34. **Van der Heijden, H. M. J. F.** Unpublished data.
35. **Vanderheijden, N., L. De Moerlooze, D. Vandenbergh, G. Chappuis, A. Renard, and C. Lecomte.** 1993. Expression of the bovine viral diarrhoea virus Osloss p80 protein: its use as ELISA antigen for cattle serum antibody detection. J. Gen. Virol. **74:**1427–1431.
36. **Van Oirschot, J. T., F. Daus, T. G. Kimman, and D. van Zaane.** 1991. Antibody response to glycoprotein I in maternally immune pigs exposed to a mildly virulent strain of pseudorabies virus. Am. J. Vet. Res. **52:**1788–1793.
37. **Van Oirschot, J. T., A. L. J. Gielkens, R. J. M. Moormann, and A. J. M. Berns.** 1990. Marker vaccines, virus protein-specific antibody assays and the control of Aujeszky's disease. Vet. Microbiol. **23:**85–101.
38. **Van Zaane, D., and M. M. Hulst.** 1987. Monoclonal antibodies against porcine immunoglobulin isotypes. Vet. Immunol. Immunopathol. **16:**23–36.
39. **Vaughn, J., R. Goodwin, G. Tompkins, and P. McCawley.** 1977. The establishment of two cell lines from the insect *Lepidoptera noctuidae*. In Vitro **13:** 213–217.
40. **Verschoor, E. J., A. L. W. van Vliet, H. F. Egberink, W. Hesselink, M. C. Horzinek, and A. de Ronde.** 1993. Expression of feline immunodeficiency virus *gag* and *env* precursor proteins in *Spodoptera frugiperda* cells and their use in immunodiagnosis. J. Clin. Microbiol. **31:**2350–2355.
41. **Vlak, J. M., A. Schouten, M. Usmany, G. J. Belsham, E. C. Klinge-Roode, A. J. Maule, J. W. M. Van Lent, and D. Zuidema.** 1990. Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology **179:**312–320.
42. **Wan, C.-H., M. I. Riley, R. R. Hook, C. L. Franklin, C. L. Besch-Williford, and L. K. Riley.** 1995. Expression of Sendai virus nucleocapsid protein in a baculovirus expression system and application to diagnostic assays for Sendai virus infection. J. Clin. Microbiol. **33:**2007–2011.
43. **Whealy, M. E., J. P. Card, A. K. Robbins, J. R. Dubin, H.-J. Rziha, and L. W. Enquist.** 1993. Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J. Virol. **67:**3786–3797.

44. **Wilson, M., and P. K. Nakane.** 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies, p. 215–224. *In* W. Knap, K. Holubar, and G. Wick (ed.), Immunofluorescence and related staining techniques. Elsevier/North Holland Biomedical Press, Amsterdam.
45. **Yao, Z., W. Jackson, B. Forghani, and C. Grose.** 1993. Varicella-zoster virus glycoprotein gpI/gpIV receptor: expression, complex formation, and antigenicity within the vaccinia virus T7 RNA polymerase transfection system. J. Virol. **67:**305–314.
46. **Zuckermann, F. A., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat.** 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62:**4622–4626.