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Tropism of Human Adenovirus Type 5-Based Vectors in Swine and Their Ability To Protect against Transmissible Gastroenteritis Coronavirus

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The infection of epithelial swine testicle and intestinal porcine epithelial (IPEC-1) cell lines by adenovirus type 5 (Ad5) has been studied in vitro by using an Ad5-luciferase recombinant containing the firefly luciferase gene as a reporter. Porcine cell lines supported Ad5 replication, showing virus titers, kinetics of virus production, and luciferase expression levels similar to those obtained in human 293 cells, which constitutively express the 5′-end 11% of the Ad5 genome. The tropism of Ad5-based vectors in swine and its ability to induce an efficient immune response against heterologous antigens expressed by foreign genes inserted in these vectors has been determined. Ad5 vectors replicate and express heterologous antigens in porcine lungs and mediastinal and mesenteric lymph nodes. Significant levels of heterologous antigen expression were also demonstrated in the small intestine (jejunum and ileum), but Ad5 replication in this organ was very poor, suggesting that Ad vectors undergo an abortive replication in the porcine small intestine. The tissues infected by Ad5 were dependent on the inoculation route. The oronasal route appeared to be best for inoculation of bronchus-associated lymphoid tissue infection, while the intraperitoneal route was best for gut-associated lymphoid tissue infection. Epithelial cells of bronchioles, macrophages, type II pneumocytes, and follicular dendritic cells were identified as targets for Ad5, while epithelial cells of the intestine were not infected by Ad5. Viruses with a deletion from 79.5 to 84.8 map units in the E3 region, with or without heterologous inserted genes, replicated to lower levels in porcine tissues than did wild-type Ad5. It was also shown that an Ad5 recombinant expressing the four antigenic sites (A, B, C, and D) of transmissible gastroenteritis coronavirus (TGEV) spike protein induced in swine immune responses which neutralized TGEV infectivity. In addition, porcine serum from Ad-TGEV-immune animals provide passive protection when mixed with fully virulent TGEV and orally administered to highly susceptible newborn piglets. These results taken together indicate that swine may be a good animal model for human Ad5 lung infection to aid in the evaluation of candidate adenovirus vaccines and that Ad5 may be suitable as a recombinant viral vaccine or for other applications in swine.

Transmissible gastroenteritis coronavirus (TGEV) infects the enteric and respiratory tissues of newborn piglets, resulting in nearly 100% mortality (48). Protection of newborn animals from TGEV infection requires the induction of secretory immunoglobulin A (IgA) in milk (8, 47). Previous studies have shown that precursors of mucosal IgA plasma cells originate in lymphoepithelial structures in the gastrointestinal and respiratory tracts (57, 58). These precursor cells switch to IgA production in gut- or bronchus-associated lymphoid tissues (GALT or BALT) and migrate to disseminated mucosal effector sites, including gastrointestinal and upper respiratory tracts, as well as exocrine tissues such as the mammary gland (50). In an attempt to develop a live virus-vectored TGEV vaccine, we have constructed recombinant human adenovirus type 5 (Ad5) vectors expressing different antigenic sites of the TGEV spike protein (56). The efficacy of such a vaccine will depend on the ability of Ad5 vectors to replicate and express heterologous antigens in porcine GALT or BALT. Previously, pigs were shown to be susceptible to infection by Ad5 (6). In the present investigation, we studied the tropism of Ad5-based vectors in swine and their ability to induce an efficient immune response against heterologous antigens encoded by a foreign gene inserted in these vectors.

The Ad5 genome consists of a double-stranded linear DNA molecule of 36 kb (20). The Ad5 replication cycle has an early phase, during which early region 1 (E1), E2, E3, and E4 transcriptional units are expressed, and a late phase corresponding to the period after the onset of viral DNA synthesis when late transcripts are expressed from the major late promoter. Late messages encode most of the viral structural proteins. E1, E2, and E4 gene products are involved in regulation of viral and cellular gene expression and in viral DNA replication and are essential for viral growth (27, 44). In contrast, E3 gene products are nonessential for viral replication in any normally permissive cell but appear to be involved in evading immune surveillance in vivo (1a, 12, 22, 24, 59). E1 and E3 and a site upstream of E4 have been used as sites for insertion of foreign DNA sequences in the generation of recombinant Ad (2, 25) by insertion of foreign DNA into the Ad5 genome, usually with compensating deletions in the E1 or E3 region. Substitution of

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E3 sequences with foreign DNA results in helper independent, nondefective vectors with a capacity for foreign genes of up to 4.9 kb (5). Ad5 recombinants containing inserts in E3 replicate in all normally permissive cell lines and may be suitable as live recombinant viral vaccines, since a number of Ad5 vectors containing inserts in E3 have been shown to express the foreign gene efficiently both in vitro and in vivo (2, 25, 39).

Several viral proteins are important for inducing an immune response to coronaviruses: the spike protein (S), the membrane protein (M), and the nucleoprotein (N) (11, 18, 52). Study of the induction of protective immunity to TGEV has focused on the S protein because it is the major inducer of TGEV neutralizing antibodies (16, 33, 37, 56) and it mediates binding of TGEV to its cellular receptor (23, 53). A correlation between the antigenic structure and the physical structure of the S protein has been established (17, 19). Four antigenic sites, named C, B, D, and A, have been identified and mapped from the amino terminus to the carboxy terminus of the S protein. Sites A, D, and B are involved in the induction of virus neutralizing antibodies (16, 33, 53). Site A is also involved in the induction of in vivo protection (14, 56), but the precise roles of the different antigenic sites in eliciting resistance to TGEV are unknown (18).

Using several Ad5 recombinants, including an Ad5-luciferase recombinant containing the firefly luciferase as a reporter gene, we have demonstrated that Ad5 vectors replicate and express heterologous antigens in porcine GALT or BALT. Epithelial cells of bronchioles, macrophages, type II pneumocytes, and follicular dendritic cells (FDCs) were identified as targets for Ad5 infection. It was also shown that an Ad5-TGEV recombinant expressing the four antigenic sites (A, B, C, and D) of TGEV spike protein induced a TGEV-specific antiserum in swine which neutralized TGEV infectivity in swine testicle (ST) cell cultures. In addition, porcine serum from Ad-TGEV-immune animals, when mixed with fully virulent virus and orally administered to highly susceptible newborn piglets, provided passive protection.

MATERIALS AND METHODS

Eukaryotic cells and viruses. The titers of Ad-derived vectors were determined and the vectors were passaged on human 293 cells, which constitutively express the 5′-end 11% of the Ad5 genome (26). 293 cells were grown in monolayer in Dulbecco modified Eagle medium supplemented with 0.01 M L-glutamine, antibiotics, and 10% fetal calf serum for cell maintenance or 5% horse serum for virus infection. The titer of TGEV was determined and the virus was passaged on epithelial ST cells (33). Intestinal porcine epithelial cells (IPEC-1), kindly provided by Helen M. Berschneider (3), were grown in monolayer in Dulbecco modified Eagle-F12 (1:1) medium supplemented with 5 μg of insulin per ml, 5 μg of transferrin per ml, 5 ng of selenium per ml, 5 μg of epidermal growth factor per ml, 0.01 M L-glutamine, antibiotics, and 5% fetal calf serum.

Ad5-*dl*309 contains a small deletion in the E3 region from Ad5 bp 30005 to 30750 (from approximately 83 to 85 map units [m.u.]) which is replaced with 642 bp of heterologous DNA that shows homology to salmon DNA. This alteration deletes all or part of the sequences coding for the E3 14.7-kDa (14.7K), 14.5K, and 10.4K proteins (4, 34). Ad-*dl*E3 contains a deletion from 79.5 to 84.8 m.u. in the E3 region (5). Ad-luc is a nondefective Ad5-luciferase recombinant containing the firefly luciferase gene flanked by simian virus 40 regulatory sequences inserted in the E3 region of the Ad5 genome (42). Ad-TS-9 is an Ad5-TGEV recombinant expressing the four antigenic sites of TGEV spike protein (56). The structures of the E3 gene region of wild-type Ad5 (Ad5-*wt*) and Ad5-based vectors used in this work are shown in Fig. 1.

The attenuated PUR46-MAD and the virulent PUR46-SW11-ST2 strains of TGEV were grown and purified and their titers were determined as described elsewhere (49).

Growth kinetics of Ad vectors in porcine cell lines. For one-step growth curves, semiconfluent monolayers of different cell lines grown in 60-mm-diameter dishes were infected at multiplicities of infection (MOIs) of 0.2, 2, and 20 PFU per cell. Virus was adsorbed for 1 h at 37°C. At 12, 24, 36, 48, and 72 h postinfection (p.i.), cell monolayers were detached with a rubber policeman, washed with cold phosphate-buffered saline, pH 7.2 (PBS), and collected by centrifugation at 3,000 rpm for 15 min at 4°C in a microcentrifuge. Cells were resuspended in 200 μl of PBS and split into 100-μl aliquots. One aliquot was used for virus titration, and the other was used to determine luciferase activity.

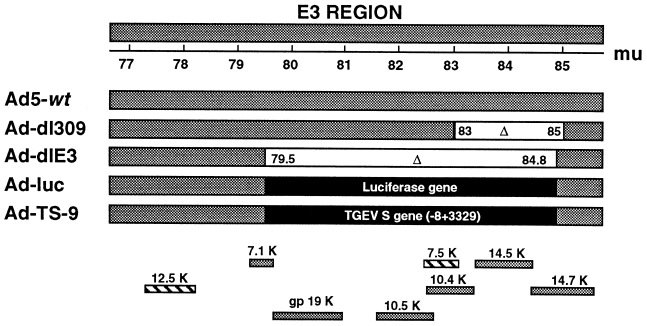

FIG. 1. Schematic representation of the E3 gene region of Ad5-based vectors used in this work. DNA sequences of Ad5-*wt* are represented by thick stippled bars. White regions indicate deleted sequences, and black regions represent heterologous sequences inserted in the deletion. Bars below the DNA sequence represent open reading frames for proteins of >6 kDa that are coded by Ad5-*wt*. Stippled bars indicate proteins that have been identified, and hatched bars indicate proteins that have been proposed.

Luciferase assays. Luciferase activity was evaluated essentially as described previously (10). Ad-luc-infected cultured cells in 100 μl of PBS were lysed by adding 900 μl of luciferase extraction buffer (1% Triton X-100, 25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol [DTT]). The lysates were pelleted by centrifugation in a microcentrifuge for 5 min at 4°C, and the supernatants were transferred to new Eppendorf tubes. One hundred microliters of undiluted or serially diluted cell extract was added to 360 μl of luciferase assay buffer (25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate [pH 7.8], 1 mM DTT, 2 mM ATP) in a luciferase assay tube (Sarstedt Inc.). Tubes containing the reaction mixture were placed in the luminometer chamber, and the enzyme reaction was initiated by the injection of 100 μl of luciferase substrate solution (0.5 mM luciferine, 25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 2 mM DTT). The light emission over the first 10 s of reaction was recorded with a Lumat LB 9501 luminometer (Berthold Systems Inc.). A background of 200 to 250 relative light units was subtracted from the actual reading before evaluation of the level of luciferase expression. The amount of luciferase in the assay samples was obtained from a standard curve generated by using known amounts of purified firefly luciferase (Boehringer Mannheim Inc.). One picogram of purified luciferase produced 790 ± 60 relative light units.

To evaluate luciferase activity in porcine tissues, 1-month-old NIH miniswine (40) were inoculated with various Ad-luc doses (from 10^8 to 10^{10} PFU per animal) by intravenous, enteric, intraperitoneal, and oronasal routes. All animals used in this study were seronegative for TGEV and for Ad5. The virus was administered in 2 ml of PBS per route. For enteric inoculation, animals were anesthetized, the abdominal cavity was opened, and virus was inoculated into the jejunum with a 25-gauge needle. Pigs were sacrificed at various times p.i., and samples from different organs were collected. Tissues were homogenized with an OMNI 2000 homogenizer (Omni International) in ice-cold PBS containing antibiotics and 1 mM phenylmethylsulfonyl fluoride to make a 1:1 (vol/vol) solution, after which 500 μl of tissue homogenates was mixed with 500 μl of luciferase extraction buffer. Tissue extracts were centrifuged for 20 min at 12,000 × *g*, and supernatants were used in luciferase assays as described above.

For virus titration, tissue homogenates were sonicated and clarified by centrifugation at 3,000 × *g*. Serial dilutions were performed in PBS and assayed by plaque formation on 293 cells.

Immunohistochemistry. Tissue samples from swine inoculated with Ad vectors were collected, cut in pieces (1 by 1 by 0.2 cm), and embedded in OCT (Miles Martín). Tissue sections were cut into 4-μm slices in a cryostat and mounted onto glass slides coated with poly-L-lysine. For histology studies, tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and cut in a microtome. Tissue sections were mounted onto glass slides coated with poly-L-lysine and stained with Carazzi hematoxylin and eosin.

For immunohistochemistry, unfixed sections were covered for 30 min with 0.15% periodic acid for endogenous peroxidase inhibition, washed with Tris-buffered saline (TBS), and incubated with the primary antibody for 1 h at room temperature. Ad5-infected cells were detected with monoclonal antibody (MAb) H2-19 (9), specific for the Ad5 72K protein, the major DNA-binding protein which is expressed early as well as relatively late in infection. Hybridoma supernatants were used at dilutions of 1:25 and 1:50. After three 5-min washes in TBS, sections were incubated for 30 min at room temperature with a 1:100-diluted biotinylated secondary antibody (goat anti-mouse; Sigma) and washed three times. Slides were incubated under the same conditions with a streptavidin-biotin complex (streptABC; Dako) for 30 min and washed three times, signal was

developed with a chromogenic substrate containing diaminobenzidine (DAB), TBS, and hydrogen peroxide for 3 to 5 min, and the mixture was then washed with water. Slides were counterstained with Carazzi hematoxylin and eosin and mounted in Depex (Serva).

For double-labeling techniques, purified MAb H2-19 was biotinylated as previously described (32) to be detected directly with strepABC complex and DAB as described above. The indirect method was then used to detect cell-type-specific antibodies. After incubation with the primary antibody, slides were incubated with alkaline phosphatase-conjugated Ig rabbit anti-mouse antibody (Dako) at a 1:40 dilution in TBS. Reactions were developed with a chromogenic substrate containing naphthol-AS-MX phosphate (Sigma), dimethylformamide, levamisol, and fast blue salt. Slides were mounted in aquamount (Gurr) without counterstaining.

Two cell-type-specific antibodies were used. MAb GM1 (DH59B; VMDR, Inc.), specific for swine monocyte/macrophage surface antigen, was used at a 1:50 dilution. To stain FDCs, a MAb specific for human CD35 antigen, which is a surface marker of human FDCs (Dako), was used at a 1:100 dilution. This MAb also binds to porcine FDCs (1).

Influence of the E3 gene on Ad5 growth. Growth kinetics were determined, both in vitro and in vivo, for several Ad5 constructs (Fig. 1) containing either no deletion of E3 sequences (Ad5-*wt*), a small deletion from 83 to 85 m.u. (Ad-*dl*309), the 1.88-kb deletion from 79.5 to 84.8 m.u. (Ad-*dl*E3), a luciferase gene inserted into the 1.88-kb deletion (Ad-luc), or an almost complete copy of the TGEV S gene inserted into the 1.88-kb deletion (Ad-TS-9). For in vitro studies, 60-mm-diameter dishes of ST cells were infected with the different viruses at an MOI of 20 PFU per cell. At 0, 12, 24, 36, 48, 72, and 96 h p.i., cell monolayers were detached, and virus titers were determined in 293 cells. For in vivo studies, 1-month-old miniswine were oronasally or intraperitoneally inoculated with 5×10^9 PFU of each virus. At 0, 12, 24, 36, 48, 72, and 96 h p.i., animals were sacrificed and virus titers were determined in lung and mediastinal lymph nodes (LN) or in small intestine and mesenteric LN, depending on whether the inoculation route was oronasal or intraperitoneal, respectively.

Immunization of swine. One-month-old swine, obtained by crossing Large White and Belgium Landrace animals, were immunized with either recombinant Ad-TS-9 or Ad5-*wt* (as a negative control). Animals were immunized via a combined inoculation route: oronasal (2×10^9 PFU per animal in 2 ml of PBS) and intraperitoneal (2×10^9 PFU per animal in 2 ml of PBS). All animals used in the experiment were free of antibodies to TGEV, porcine respiratory coronavirus (PRCV), and Ad5, as tested by radioimmunoassay (RIA) and neutralization assays (49). Swine infections were performed in a containment facility with a P3 safety level. Groups of four animals infected with different Ad-TGEV recombinants were maintained in different safety rooms. Animals immunized with the same recombinant were not isolated from each other. Sera and saliva were collected at 0, 4, 6, 8, and 10 weeks p.i. and were evaluated by RIA, enzyme-linked immunosorbent assay (ELISA), and neutralization against TGEV. Ten weeks p.i., all animals were euthanized and bled in order to prepare a large quantity of serum for in vivo protection experiments.

RIA, ELISA, and serum neutralization assays. The antibody response elicited in swine immunized with recombinant Ad-TS-9 was studied by RIA, ELISA, and TGEV neutralization assays. RIA was performed with purified TGEV as the antigen (0.1 μg per well) as previously described (33). IgA and subclass IgG responses against TGEV were characterized by ELISA with purified TGEV as the antigen (0.1 μg per well) as previously described (15). The porcine Ig class-specific MAbs, anti-IgA, anti-IgG1, and anti-IgG2, were kindly provided by C. R. Stokes (Bristol University, Bristol, England). RIA and ELISA titers were defined as the inverse of the highest dilution giving a binding threefold higher than the background level. Neutralization of TGEV was performed by incubating serial 10-fold dilutions of the virus with a 1/20 dilution of the antiserum at 37°C for 30 min, and the virus-antibody mixture was plated on ST cells as previously described (13). The neutralization index was defined as the $\log_{10}$ of the ratio of the PFU after incubation of the virus in the presence of control serum or in the presence of the indicated antiserum.

Protection of swine by immune serum. Porcine antiserum elicited by recombinant Ad-TS-9 was collected 10 weeks p.i. and used to study its ability to protect against TGEV in vivo. The virulent TGEV strain PUR46-SW11-ST2 (10^7 PFU per swine) (49) was mixed with 3 ml of the porcine antiserum elicited by recombinant Ad-TS-9, incubated at 37°C for 60 min, and administered by using a gastric tube to 2-day-old miniswine born to TGEV-seronegative sows. Inoculated animals were fed three times per day with milk formula for newborns (Nidina 1; Nestlé) containing 3 ml of the antiserum. Control animals were treated by the same procedure but with serum induced by Ad-*dl*309. Five animals per group were challenged, and control serum or antiserum was administered following the challenge every day until the animal either died or was sacrificed. TGEV titers were determined after 1, 2, and 3 days in animals challenged with virus incubated in the presence of control serum or after 1, 2, and 5 days p.i. in animals challenged with virus incubated with immune serum, using tissue extracts from the jejunum and ileum and from the lung. Animal sampling was interrupted in the control group when all the animals died. The last antiserum dose was given to piglets 12 h before sample collection to avoid the presence in the enteric tissues of antibody from external sources. Furthermore, the absence of the antibody in the enteric tissues was proven by showing that the tissue extracts did not neutralize samples with a known virus titer. Tissue homogenization was performed at 4°C with an OMNI 2000 homogenizer (Omni International).

RESULTS

Replication of Ad5 vectors in porcine cell lines. The recently generated helper-independent recombinant Ad5-luc provides a very sensitive assay for gene expression in human cell lines infected with the vector (42). Because of the high levels of luciferase expression observed with this vector, it was used to study Ad-mediated gene expression in porcine cells. Dose responses and time courses of Ad-luc infection in two porcine cell lines and the human cell line 293 were determined (Fig. 2). Infection of porcine cells was assessed both by evaluating luciferase reporter gene expression and by determining virus production at 12, 24, 36, 48, and 72 h p.i. High levels of luciferase activity were obtained, and dilutions of cell extracts were made to maintain assays in the linear range of the luciferase assay. Human and porcine cell lines showed similar levels of luciferase activity (Fig. 2A, C, and E) and of virus yields (Fig. 2B, D, and F). Luciferase activity and virus titer were dose and time dependent and were near maximal by 48 to 72 h p.i. These results indicate that human Ad5 can replicate efficiently in porcine cells and that an Ad5 vector with a reporter gene in E3 can express the cloned cDNA at high levels in porcine cells.

In vivo Ad5 vector replication and Ad-mediated gene expression in porcine tissues. Recombinant Ad-luc was used to investigate the process of virus dissemination and the level of foreign gene expression in porcine tissues. Four-week-old minipigs were inoculated with Ad-luc (10^9 PFU per animal) by various routes (intravenous, enteric, intraperitoneal, and oronasal). Four animals per group were inoculated, and luciferase expression and virus titers were determined in various organs at 24 h p.i. (Fig. 3). Organ distribution of luciferase activity and of virus recovered was dependent on the inoculation route. Highest levels of luciferase were observed when virus was inoculated by oronasal and intraperitoneal routes, while infection by intravenous and enteric routes resulted in luciferase expression and virus recovery levels that were drastically reduced or undetectable, depending on the tissue (in our system, the detection limits were 5 pg of luciferase and 10^1 PFU/g of tissue). The organs expressing highest levels of luciferase and virus after oronasal inoculation were the lung and mediastinal LN. By contrast, in animals intraperitoneally inoculated, highest levels of luciferase expression were detected in the small intestine and mesenteric LN, but the level of virus recovered in the small intestine was very low.

Since highest levels of luciferase expression were detected in animals inoculated oronasally and intraperitoneally, these routes were selected in subsequent experiments. The dose dependence and time course of Ad-luc infection in lungs, small intestines, and mediastinal and mesenteric LN from animals inoculated simultaneously by both oronasal and intraperitoneal routes were studied by using virus doses ranging from 10^8 to 10^{10} PFU per animal (Fig. 4). Ad-mediated luciferase expression (Fig. 4A, C, and E) and virus titers (Fig. 4B, D, and F) were dose and time dependent in the four organs studied. The highest levels of both luciferase expression and virus titer were obtained in the lung. Luciferase activity was detected in this organ as early as 15 h p.i., reaching a maximum at about 2 days p.i. and declining thereafter. The same profiles but with a lower level of infection and expression were found in mediastinal and mesenteric LN (Fig. 4). However, a different time course profile was found in the small intestine: luciferase activity was maximal at 24 h p.i. and then declined more rapidly than in the

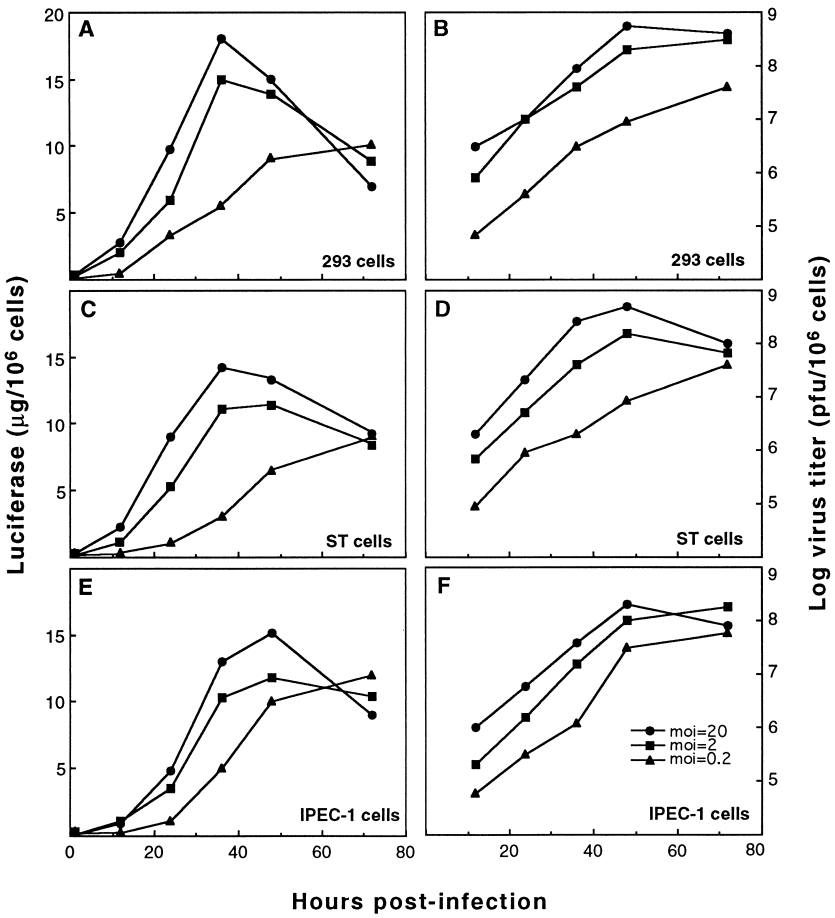

FIG. 2. Dose responses and kinetics of Ad-luc infection in porcine and human 293 cell lines. Semiconfluent monolayers of 293, ST, and IPEC-1 cells were infected with Ad-luc at MOIs of 0.2, 2, and 20. Cell monolayers were collected at 12, 24, 36, 48, and 72 h p.i., washed, and then resuspended in PBS. Luciferase activities (A, C, and E) and virus titers (B, D, and F) were determined as described in Materials and Methods. Each point represents the mean of four values determined in two different experiments. Standard deviations were lower than 25% in all cases and are not represented.

other three organs. Furthermore, virus titer level in this organ was very low.

Cell types infected by Ad5-based vectors in vivo. To determine the number and cell type in the target organs infected by Ad5, 4-week-old minipigs were inoculated with different doses of Ad5 vectors (10^8 to 10^{10} PFU per animal) by various routes (intravenous, enteric, intraperitoneal, and oronasal). At different times p.i., tissue samples from inoculated and mock-infected (negative control) animals were collected. Several (more than four) tissue samples from the different regions of each organ and its corresponding draining LN were collected from two different animals per condition. Tissue sections were stained by using biotinylated MAb H2-19, specific for the Ad5 72K protein (9), and an indirect avidin-biotin technique, and signal was developed with DAB. Immunohistochemistry revealed positive staining for MAb H2.19 in animals infected with doses higher than 10^9 PFU of Ad5 vector per animal (Fig. 5). The fraction of positive cells was directly proportional to the input dose (results not shown). When the oronasal route was used, positive cells were detected in the lung and its corresponding draining mediastinal LN, while other organs, such as the digestive tract, were consistently negative. In contrast, after intraperitoneal inoculation, MAb H2-19-positive cells were detected in the small intestine (particularly in the ileum), gut-associated lymphoid structures such as Peyer's patches, and mesenteric regional LN. After inoculation of the virus by both oronasal and intraperitoneal routes, positive staining was found in the lung, ileum, and mediastinal or mesenteric LN. After inoculation by the intravenous and enteric routes, no positive cells were detected (results not shown). The absence of positive cells in some tissues showing low but significant levels of both luciferase expression and virus titer may be due to the lower sensitivity (at least 20-fold) of the immunochemistry assay, related to the luciferase assay and virus titration.

The numbers of Ad5-positive cells in enteric or respiratory tissues were highest at 2 days p.i., decreasing at 4 days p.i. In the lung, positive staining with MAb H219 was localized mainly in epithelial cells of bronchioles (Fig. 5A), which showed a strong nuclear staining. Positive cells were also observed in alveoli (Fig. 5B). To identify the nature of these cells, double staining was performed with Ad-specific MAb H2-19 and MAb DH59B, which is specific for monocytes/macrophages and granulocytes. Moderate numbers of alveolar cells stained with H2-19 MAb (dark brown) in the cell nucleus. Some alveolar cells showed double staining with MAb DH59B (blue) on the membrane and with H2-19 MAb (brown) inside the cell. On the basis of their size, absence of apparent granulation, and absence of segmented nuclei, we concluded that the double-stained cells were monocytes/macrophages (and not granulocytes). In contrast, many alveolar lining cells showed positive

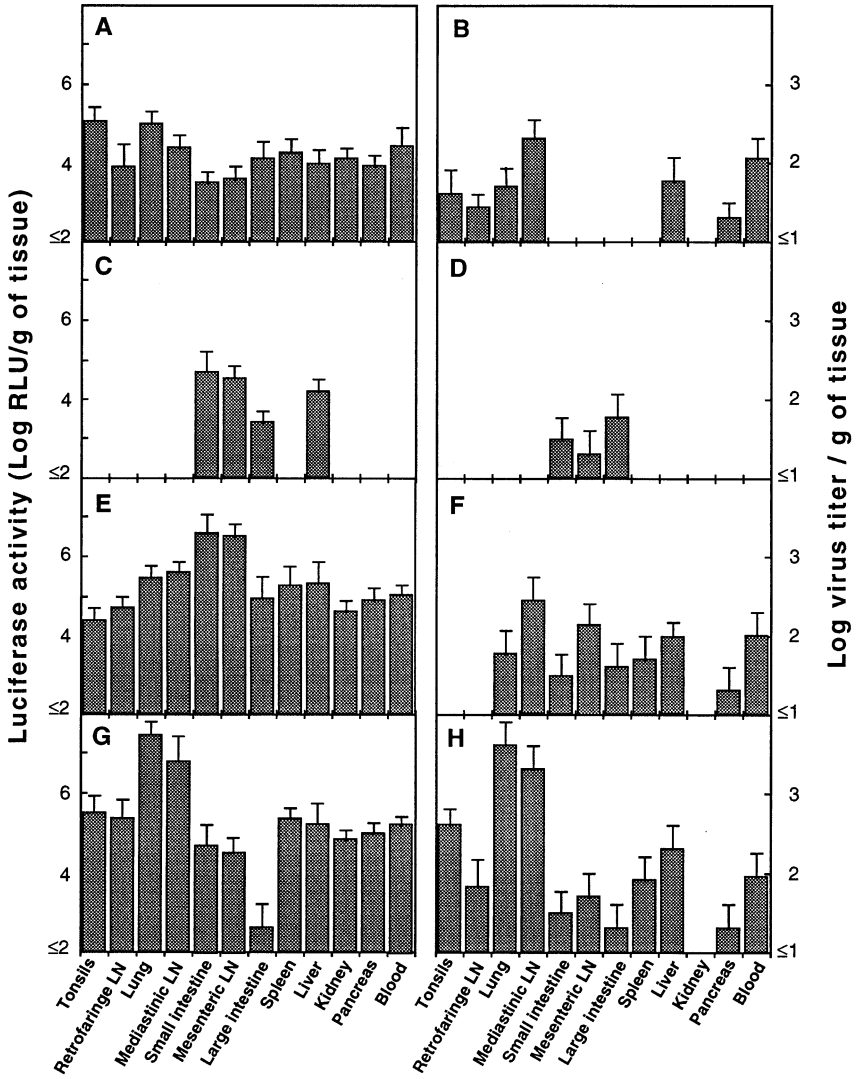

FIG. 3. Ad-luc recombinant virus replication and luciferase expression in porcine tissues after inoculation by different routes. Four- to five-week-old minipigs were inoculated with 10^9 PFU of Ad-luc via intravenous (A and B), enteric (C and D), intraperitoneal (E and F), and oronasal (G and H) routes. At 24 h p.i., animals were sacrificed and homogenates from different organs were prepared as described in Materials and Methods. Luciferase activities (A, C, E, and G) and virus titers (B, D, F, and H) were determined. Each bar represents mean results for at least four animals ± standard deviation. RLU, relative light units.

staining with the Ad5-specific MAb but were negative for macrophage markers, suggesting that these cells were type II pneumocytes (Fig. 5B). In lymph nodes (Fig. 5C and D), the target cells were mainly FDCs, localized in germinal centers of either mediastinal or mesenteric LN. The identification of FDCs as targets for Ad5 was based in two criteria: staining by MAb CD35 (result not shown) and morphology (dendritic shape with long processes which shows a sharply defined dense meshwork in B-cell follicles, a morphology not shown by B cells, macrophages, or granulocytes). Positive staining by the Ad5-specific MAb was also found in a minor proportion of scattered macrophages located in LN sinuses. In the small intestine (Fig. 5E and F), epithelial cells were negative, and only scarce positive macrophages could be detected in the lamina propia. In Peyer's patches, there were some positive dendritic cells.

Histopathology revealed that lung pneumonia developed in animals infected with Ad-*wt* or Ad-*dl*309 (Fig. 1) by the mixed oronasal and intraperitoneal route. Other organs, such as the spleen, liver, and intestine, showed only nonspecific changes. Lobular interstitial pneumonia with a moderate bronchiolar component was present at 24 h p.i., but it tended to spontaneously resolve beginning 96 h p.i. and was resolved by week 1 p.i. Interestingly, animals infected with viruses containing the 1.88-kb E3 deletion (Ad-*dl*E3) or a foreign gene inserted in the E3 deletion (Ad-luc and Ad-TS-9; Fig. 1) developed a lower degree of pathologic changes and fewer pneumonic areas.

Influence of the E3 gene on Ad5 growth in porcine cells in vitro and in vivo. Since the Ad5 recombinants used in this work have 1.88 kb deleted from the Ad5 E3 gene, we studied whether the introduction of a deletion or of an insertion would affect in vitro and in vivo virus replication. Growth kinetics were determined for Ad5-*wt*, Ad-*dl*309, Ad-*dl*E3, Ad-luc, and Ad-TS-9. For in vitro studies, ST cells were infected at an MOI of 20 PFU per cell. At various times p.i., cell monolayers were detached, and virus titers were determined in 293 cells. For in vivo studies, 1-month-old miniswine were oronasally inoculated with 5×10^9 PFU of each virus. Animals were sacrificed at various times p.i., and virus titers in the lung were deter-

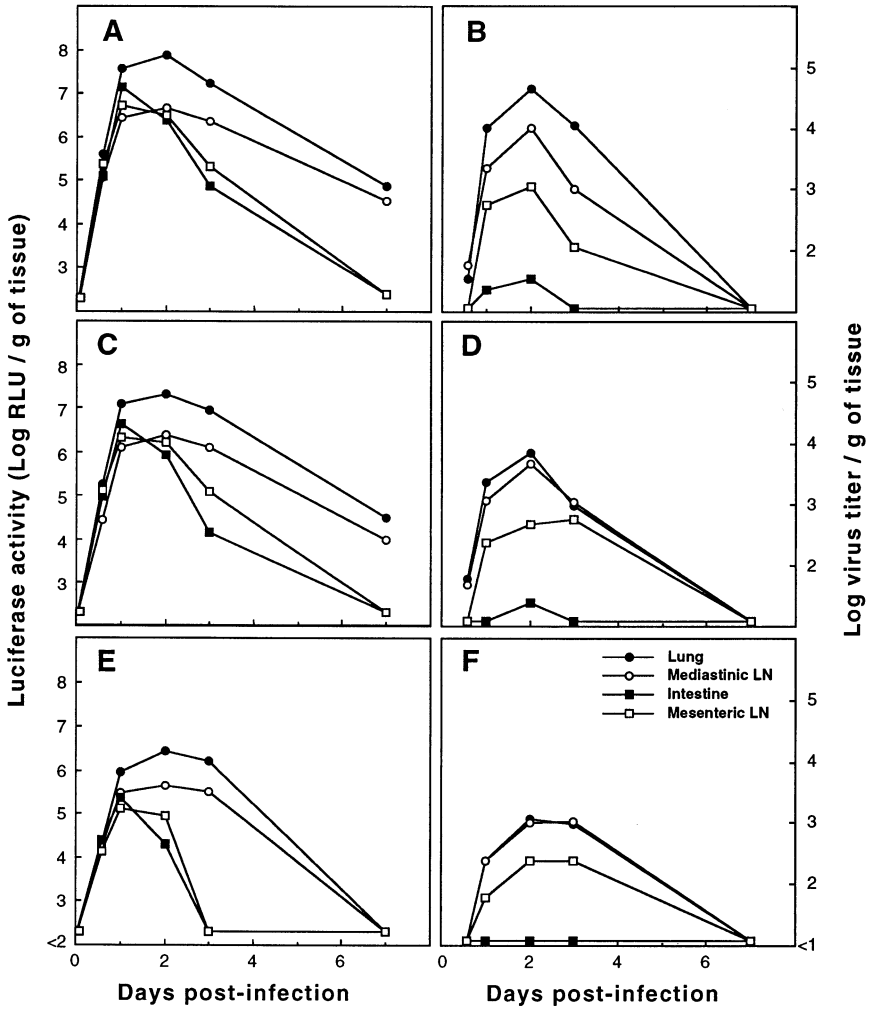

FIG. 4. Dose responses and time courses of Ad-luc infection in porcine tissues. Groups of 10 minipigs were inoculated simultaneously by both oronasal and intraperitoneal routes with Ad-luc at 10^{10} (A and B), 10^9 (C and D), and 10^8 (E and F) PFU per animal. At different times p.i., two animals from each group were sacrificed, and homogenates from the lung, mediastinal LN, small intestine, and mesenteric LN were prepared as described in Materials and Methods. These homogenates were used for luciferase activity assays (A, C, and E) and for virus titer determination (B, D, and F). Each point represents the mean of four values determined in two different experiments. Standard deviations were lower than 25% in all cases and are not represented. RLU, relative light units.

mined. The statistical analysis of the results by Student's *t* test indicated nonsignificant differences between virus growth curves in ST cultured cells (Fig. 6A), and final yields of viruses with the 1.88-kb E3 deletion with or without inserts were always in the range of those obtained for Ad5-*wt* or Ad-*dl*309. Other authors (5) have reported similar relative growth of the different recombinants in human 293 cells. However, the results of the in vivo experiments (Fig. 6B) showed a significant decrease (10-fold) in virus recovery in the lungs from animals inoculated with viruses containing the 1.88-kb E3 deletion (Ad-*dl*E3, Ad-luc, and Ad-TS-9) compared with those inoculated with Ad-*wt* or Ad-*dl*309. The degree of significance of these differences was higher than 99.99% ($P < 0.01$) throughout the time course. Similar results were found in mediastinal and mesenteric LN; in the small intestine, these differences were less marked (nonsignificant), probably because of the poor replication in this organ.

Induction of a TGEV-specific immune response in swine by recombinant Ad-TGEV. To determine whether a single inoculation of swine with an Ad5 recombinant was an efficient inducer of systemic and secretory immune responses against heterologous antigens, recombinant Ad-TS-9, expressing the four antigenic sites of TGEV spike protein (56), was used. A group of four swine was immunized with this recombinant, and serum and saliva were collected at several times p.i. and tested for TGEV antibodies by RIA, by ELISA, and by neutralization (Fig. 7). All animals used in the experiment were free of antibodies to TGEV, PRCV, and Ad5, as tested by RIA and neutralization assays. During the performance of the experiments, the animals were not infected by PRCV from external sources, as shown by the lack of seroconversion to PRCV-positive animals in the control group vaccinated with the Ad5 vector without insert (Fig. 7). At 6 weeks p.i., the anti-TGEV RIA titers in sera from Ad-TS-9-immunized swine were higher than 10^3 and the neutralization index was higher than 5. Both RIA titer and neutralization index showed an increase at 8 weeks p.i. (RIA titer of 2×10^4 and neutralization index of 6), maintaining these values at 10 weeks p.i. Lower but significant levels of TGEV-specific antibodies were also detected by RIA (Fig. 7A) and neutralization (Fig. 7B) in saliva. In addition, high ELISA titers of TGEV-specific IgA were found in serum and saliva collected 10 weeks p.i. (Fig. 7C).

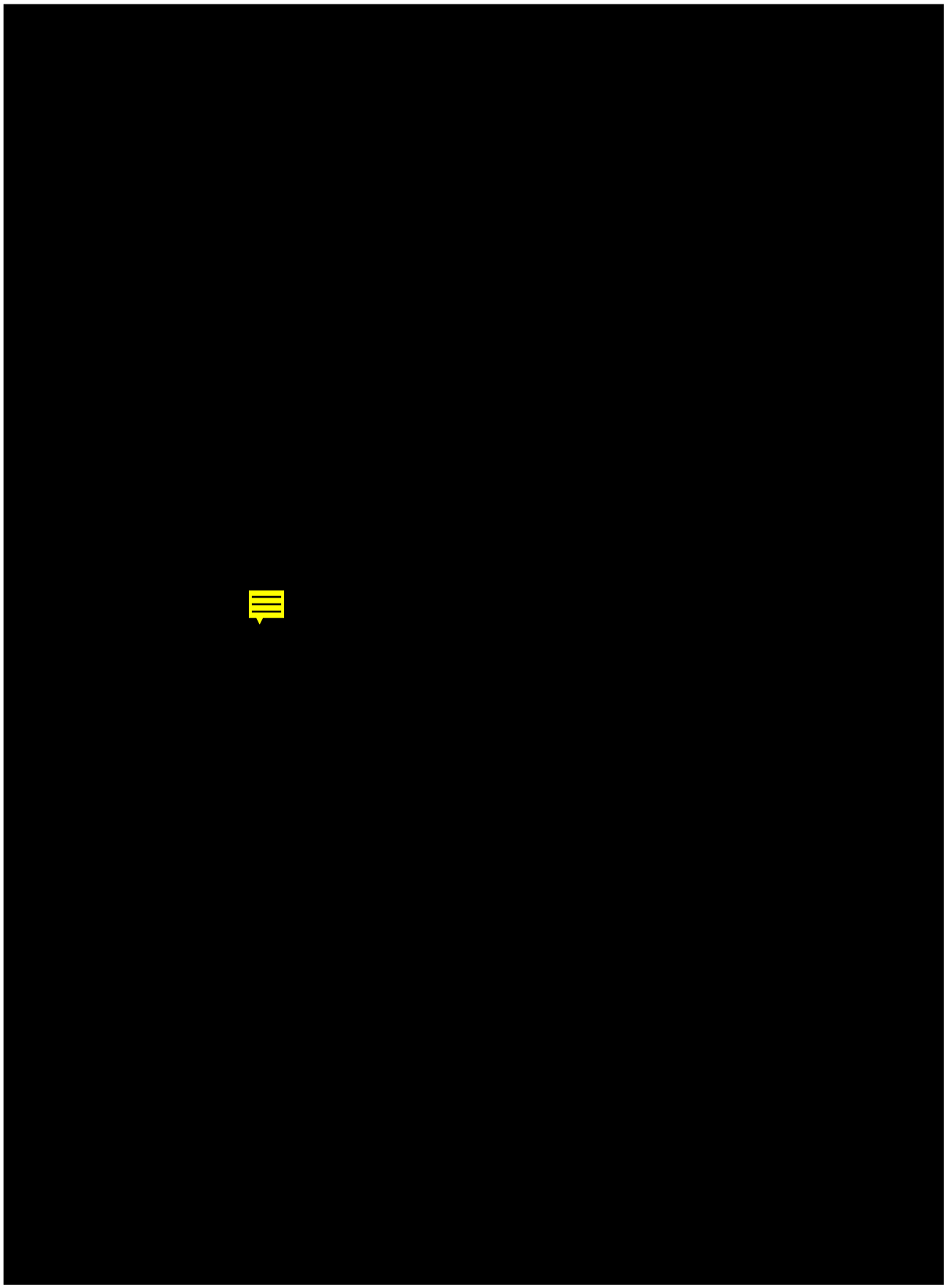

FIG. 5. Immunohistochemical detection of Ad-infected cells in swine. Animals were inoculated oronasally and intraperitoneally with 5×10^9 PFU of Ad5-*wt* by each route. (A) Swine bronchiole stained by using biotinylated MAb H2-19 and an indirect avidin-biotin technique, developed with DAB. There is strong nuclear staining of epithelial lining cells (indicated by arrows) of bronchioles, which represented the most frequent infected cell type. Magnification, ×208. (B) Swine alveoli stained by using biotinylated MAb H2-19 and a direct technique, developed with DAB (brown staining). Surface blue staining was obtained with antimacrophage antibody GM1 and indirect phosphatase labeling. A moderate number of alveolar cells stained dark brown in the cell nucleus. Some alveolar cells double stained blue on the membrane and brown inside the cell, corresponding to infected alveolar macrophages (indicated by arrows). In contrast, other lining cells showed positive staining to viral antigens but were negative to macrophage markers (arrowhead), suggesting that those elements were type II pneumocytes. In the microscopic analysis, bronchiolar cells and pneumocytes exhibited nuclear inclusion bodies of different sizes. Magnification, ×208. (C) Swine mesenteric LN stained with MAb H2-19. Positive staining displayed a typical distribution in the germinal center areas, corresponding to FDCs. Positive staining by the Ad5-specific MAb was also found in a minor proportion of scattered macrophages located in LN sinuses. Magnification, ×83. (D) Detail of FDCs in germinal centers stained with an Ad5-specific MAb at different magnifications: D1 and D2, ×208; D3, ×332; D4, ×830. FDCs showed positive staining with MAb H2-19 not only in the nucleus but also in the cytoplasmic processes and extracellular surrounding space. (E) Panoramic view of ileum stained with MAb H2-19. Dispersed H2-19-positive cells were found in submucosal lymphoid tissues and in Peyer's patches (PP) (indicated with arrows). Note the negativity of epithelial cells lining the intestinal villi (V) and crypt (C). Magnification, ×83. (F) Detail of positive cells located in submucosal lymphoid tissues (F1) and in the lamina propia (F2). Magnification, ×332.

To study the potential of the antiserum elicited by Ad-TS-9 for in vivo protection against TGEV, serum induced by this recombinant was analyzed for its ability to prevent TGEV infection. The challenge was performed with virulent TGEV (PUR46-SW11-ST2 strain; 10^7 PFU per dose) mixed with the antibody induced by Ad-TS-9. Virus-antibody mixtures were incubated at 37°C for 60 min and administered to highly susceptible 2-day-old miniswine. TGEV titers were determined in the jejunum and ileum and in the lung at 1, 2, 3, and 5 days p.i. The results (Table 1) indicated that low titers ($<5 \times 10^2$ PFU/g of tissue) of infectious virus were detected in the small intestines of newborn pigs when they were administered the antibody elicited by recombinant Ad-TS-9, while titers ranging between 10^5 and 10^7 PFU/g of tissue were detected in the tissues of control animals to which serum induced by Ad5-*wt* used as a control was administered. In addition, neither mortality nor clinical symptoms were observed in animals treated with serum induced by recombinant Ad-TS-9, while control animals exhibited diarrhea 24 to 30 p.i. and died around day 3 p.i. (Table 1).

DISCUSSION

Various types of Ad are very useful mammalian cell expression vectors with potential utility as live recombinant vaccines, in gene therapy, or for high-level protein production (25, 28, 29). An Ad-based vaccine containing unattenuated Ad4 and Ad7 has been used with satisfactory results for more than a decade to immunize U.S. military recruits against acute respiratory infections (41, 54). It would be useful to have an animal model to aid in the evaluation of candidate Ad vaccines but, unfortunately, human Ad types have a restricted host range. In general, infection of cultured cells of other species results in much lower or no virus production compared with human epithelial cells. Rhesus monkey cells are nonpermissive for Ad5 replication, while cell lines derived from African green monkeys are generally semipermissive (36). Human Ad 4, Ad5, Ad21, and Ad31 have been reported to undergo abortive replication in canine cells (45). Among rodents, hamsters, and cotton rats have been proposed as suitable models for respiratory infection by human adenovirus (22, 30, 46). Hamster cells are permissive for Ad2 and Ad5 replication (35) but are significantly less permissive than human cells, while mouse cells are semipermissive (7, 51). In this study, we found that porcine cell lines support Ad5 replication, showing virus kinetics and virus titers similar to those obtained for human epithelial cells. In addition, the virus expression levels of heterologous genes inserted in the E3 region of Ad5 detected in porcine cells were as high as in human epithelial cells.

Since Ad-luc replication and Ad-mediated expression of heterologous genes were very efficient in two porcine cell lines, we investigated whether this finding could be extended to in vivo studies. Recombinant Ad-luc, expressing luciferase as a reporter gene, was used (42). The results indicated that swine was susceptible to Ad5, the infection of the different organs being dependent on the inoculation route. The oronasal route

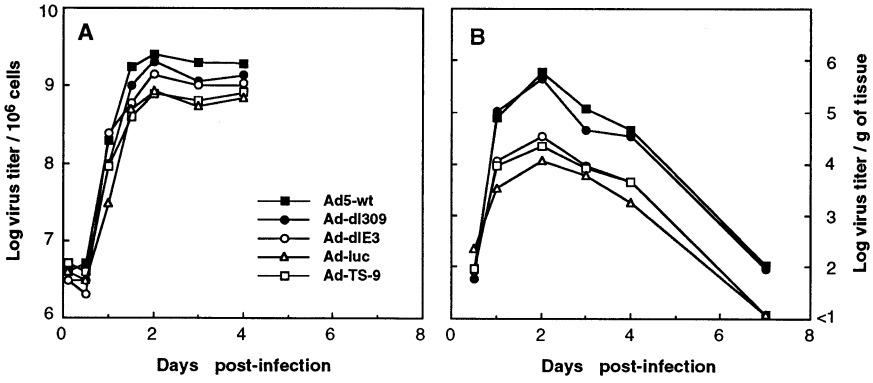

FIG. 6. One-step growth curves comparing the growth kinetics of Ad5 with deletions and insertions in the E3 gene with those of Ad5-*wt*. For in vitro studies (A), ST cells growing on 60-mm-diameter dishes were infected at an MOI of 20 PFU per cell. At the indicated times p.i., cell monolayers were detached, and virus titers were determined on 293 cells. For in vivo studies (B), 1-month-old miniswine were oronasally and intraperitoneally inoculated with 5×10^9 PFU. At the indicated times p.i., animals were sacrificed, and virus titers in lung were determined on 293 cells. Ad-*dl*309 and Ad-*dl*E3 contain deletions from 83 to 85 m.u. and from 79.5 to 84.8 m.u., respectively. Ad-luc and Ad-TS-9 contain the luciferase gene and a 3,329-nucleotide fragment of the S gene of TGEV, respectively, replacing the 1.88-kb deletion (Fig. 1). Each point represents the mean of four values determined in two different experiments. Standard deviations were lower than 25% in all cases and are not represented. Student's *t* test was used to assess differences among the viruses; the degree of significance of the observed differences was higher than 99.99% ($P < 0.01$) throughout the time course.

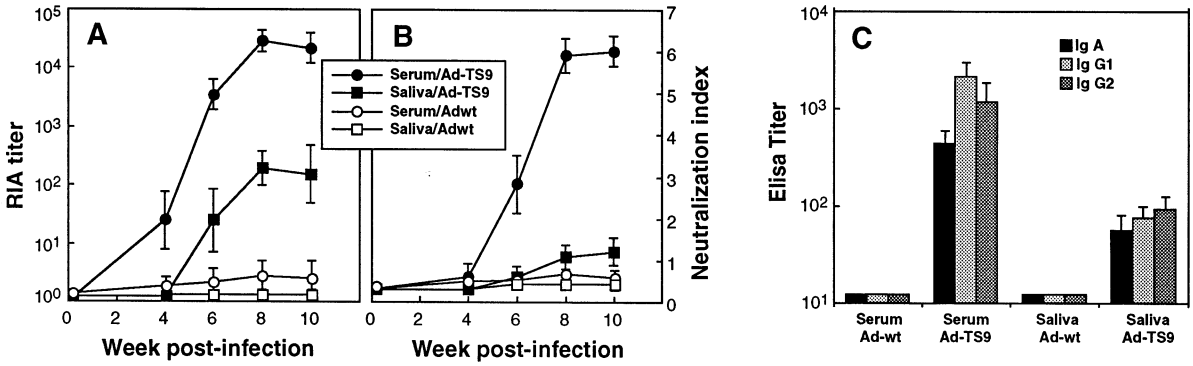

FIG. 7. Immune response induced by an Ad-TGEV recombinant in swine. One-month-old swine were immunized with recombinant Ad-TS-9, expressing the four antigenic sites of TGEV spike protein, or with Ad-*wt* as a negative control. The virus (2×10^9 PFU/2 ml) was administered by oronasal and intraperitoneal routes simultaneously. Sera and saliva were collected at 0, 4, 6, 8, and 10 weeks p.i. and evaluated by RIA (A) and neutralization (B) against TGEV. In samples obtained at 10 weeks p.i., the different Ig isotypes (IgA, IgG1, and IgG2) induced against TGEV were also determined by ELISA, using purified TGEV as the antigen and porcine Ig class-specific MAbs (C). The mean value from four animals and standard deviation are represented for each time point. RIA and ELISA titers and the neutralization index were determined as described in Materials and Methods.

appeared to be best for respiratory infection. Virus recovery and luciferase expression levels in lung and mediastinal LN from animals inoculated by the oronasal route were at least 20-fold higher than in the other organs studied. The intraperitoneal route provided the best results for enteric tissue infection. Although Ad5 replication in the small intestine was very low or absent, in other enteric tissues, such as mesenteric LN, high replication levels were found. In animals inoculated by this route, highest levels of luciferase expression were detected in the small intestine and mesenteric LN, although virus recovery from the small intestine was very poor. Other inoculation routes studied (enteric and intravenous) were much less efficient in infecting all organs. Considering the combined results from all inoculation routes, we concluded that replication of Ad5 in porcine tissues other than the lung, small intestine, and mediastinal and mesenteric LN was very low or absent. In these tissues, low levels of luciferase activity were expressed, consistent with the low virus levels detected, which probably represent residual input virus. Consequently, the dose response and kinetics of Ad5 infection were determined in the lung, small intestine, and mediastinal and mesenteric LN from animals inoculated simultaneously by the oronasal and intraperitoneal routes. These experiments showed that the magnitudes of Ad5 replication and of Ad5-mediated luciferase expression were directly proportional to the input dose of virus. Results similar to those obtained for swine in this study were also observed in Ad5-infected cotton rats (46). This observation is at variance with the results from earlier animal studies using influenza virus and pneumonia virus of mice, in which a lower input dose delayed the attainment of the viral titer peak but did not diminish the magnitude of that titer (21, 31). Results with Ad-luc in the lung and in mediastinal and mesenteric LN suggest that even though Ad5 multiplies, it undergoes only a few cycles of replication in these tissues, and infected cells are probably eliminated before extensive virus dissemination. This hypothesis is also supported by the short duration of virus production and gene expression observed in kinetic experiments, in which the peak was attained 2 to 3 days p.i., expression and virus yields declining thereafter. A different situation was observed in the small intestine, in which infectious virus likely represents residual input virus rather than newly replicated virus progeny. The apparent discrepancy between the high level of antigen expression in the small intestine and the absence of vector replication in this organ suggests that the Ad5 vector may enter some cells from the small intestine and express the antigens encoded by heterologous gene, but no secondary infection occurs; i.e., the Ad5 vector undergoes abortive replication in the porcine small intestine. Since the Ad-luc virus was directly inoculated into the intestine, it can be excluded that the virus was inactivated by passage through any other organs, such as the stomach.

TABLE 1. Protection of swine with porcine serum elicited by an Ad-TGEV recombinant

Treatment[a]	Days postchallenge	No. survivors	Virus recovery (log virus titer)	
			Lung	Intestine
Serum elicited by Ad-TS-9	1	5	≤1	2
	2	5	≤1	1.2
	5	5	≤1	2.5
Serum elicited by Ad-*wt*[b]	1	5	6	5.2
	2	3	6.2	5.5
	3	0	6.5	5.6

[a] Antisera were incubated in vitro with virulent TGEV (1 h at 37°C) prior to baby pig challenge.
[b] Negative control.

Immunohistochemistry results were consistent with those obtained for luciferase expression and virus replication. This approach allowed the determination of the cell type susceptible to Ad vectors in swine. In the lung, viral antigens were detected mainly in epithelial bronchiolar cells, type II pneumocytes, and alveolar macrophages. In the small intestine, there were scarce positive cells in the lamina propia and some positive FDCs in Peyer's patches. Infection of small intestine epithelial cells was never detected. The lack of susceptibility of this cell type to Ad5 could explain the absence of infection when the enteric route is used. Even though intestinal epithelial cells were not infected, there was another interesting finding with respect to the induction of an immune response: a selective tropism of Ad5 vectors for FDCs of LN from lung and intestine draining areas. FDCs have a crucial role in the generation of a long-term humoral immune response (38, 55). All of these data and the high humoral immune response induced by Ad5 in swine (reference 56 and Table 1) suggest that Ad5 may be a suitable vector in swine.

Histopathology studies showed that lung pathology was markedly less after inoculation with Ad5 recombinants than after inoculation with Ad5-*wt*. This observation is of particular interest since these recombinants contain a large E3 deletion (from 79.5 to 84.8 m.u.). The E3 gene complex codes for several products, and although their functions are not fully understood, it is known that this region is not essential for virus replication. Two of the proteins encoded by E3 (14.7K and gp19) have been implicated in the protection of virus-infected cells from immune destruction (12, 24, 59). Coincident with our results, other authors showed that recombinants containing a deletion of the sequence coding for gp19 did not cause enteric disease in chimpanzees (43). In vivo experiments showed a lower level of replication in the lung for viruses with the 1.88-kb E3 deletion, with or without a heterologous inserted gene (Ad-*dl*E3, Ad-luc, and Ad-TS-9), than for Ad-*wt* or Ad-*dl*309. Differences in pathology seem to correlate with differences in replication efficiency. Since the replication capacity of Ad-*dl*309 (containing a small E3 deletion from 83 to 85 m.u.) was not affected, the decrease in replication and pathogenicity observed in Ad5 with larger E3 deletions could be mapped between 79.5 and 83 m.u. on the Ad5 genome. Thus, the E3 functions responsible for these differences were likely to be gp19 and/or the 10.5K protein. Elucidation of the precise E3 functions responsible for this effect requires further studies, and swine may be a good model for such analyses.

The results presented in this report indicate that Ad5-based vectors infect several porcine tissues and can express high levels of foreign genes, suggesting that an efficient immune response against antigens expressed by Ad vectors may be attainable. A single inoculation with Ad-TS-9, which expressed the globular part of TGEV spike protein, was sufficient to elicit in serum high levels of anti-TGEV antibodies which persisted for at least 10 weeks. These antibodies neutralized TGEV infectivity with a high neutralization index. Lower but significant levels of anti-TGEV antibodies were also elicited in saliva. In addition, significant levels of TGEV-specific IgA were found in serum and saliva samples collected 10 weeks p.i., showing that IgA contributes to the response against TGEV induced by the recombinant Ad-TGEV.

The late response induced by the Ad5 vectors in swine is surprising. A transient virus-induced immunosuppression cannot be excluded, but it seems unlikely because Ad5 infection is quickly controlled by the pig immune system and the induced pathogenesis tended to spontaneously resolve in a few days. Seven days after inoculation of a high Ad5 dose, neither virus replication nor histopathologic alteration was found in any tissue. In addition, infection of swine tissues does not seem to compromise the swine immune system, as shown by the strong immune response elicited by these vectors against Ad5 and the heterologous antigens (45, 56). The antibody response induced by Ad-TS-9 in swine was higher than that induced in hamsters and was more stable than that obtained with similar constructs expressing a longer insert of TGEV sequences (56). In addition, serum elicited in swine by this recombinant prevented the in vivo replication of virulent TGEV administered orally as virus-antibody mixtures and fully protected highly susceptible piglets from clinical signs and death. Protection of newborn animals from TGEV infection requires the induction of lactogenic immunity. Although further experiments with recombinant Ad-TS-9-immune pregnant sows are required to test the protection of neonates through lactogenic immunity, Ad5 vectors have a high probability of inducing effective mucosal immunity against TGEV, since these vectors showed tropism for BALT and to a minor extension for GALT. Final demonstration of the potential of Ad5 vectors expressing TGEV antigens to protect swine against TGEV or other enteric or respiratory pathogens will require the performance of challenge experiments in Ad5 vector-inoculated pigs. In addition, the selective tropism for FDCs of LN from lung and intestine draining areas suggests that immunity elicited by these vectors could persist for long periods. In fact, immunization of hamsters with recombinant Ad5-TGEV elicited an immune response that lasted at least for 4 months (56). In addition, it has been shown (Fig. 7) that in swine, the immune response elicited by recombinant Ad-TS9 lasted for at least 10 weeks p.i.

The Ad vector-induced pathologic effect was localized, taking the form of a self-limiting pneumonia which tended to spontaneously resolve in a few days. These results, and the fact that no clinical symptoms were found in any of the more than 50 animals inoculated with a high dose of Ad recombinants during the 10 weeks following inoculation, suggest that this vector could be used as a live vaccine in swine without secondary complications associated with the vector. Nevertheless, concurrent infection of swine by Ad vectors and opportunistic infections by other respiratory pathogens may pose safety risks that should be evaluated before these vectors are used in field conditions.

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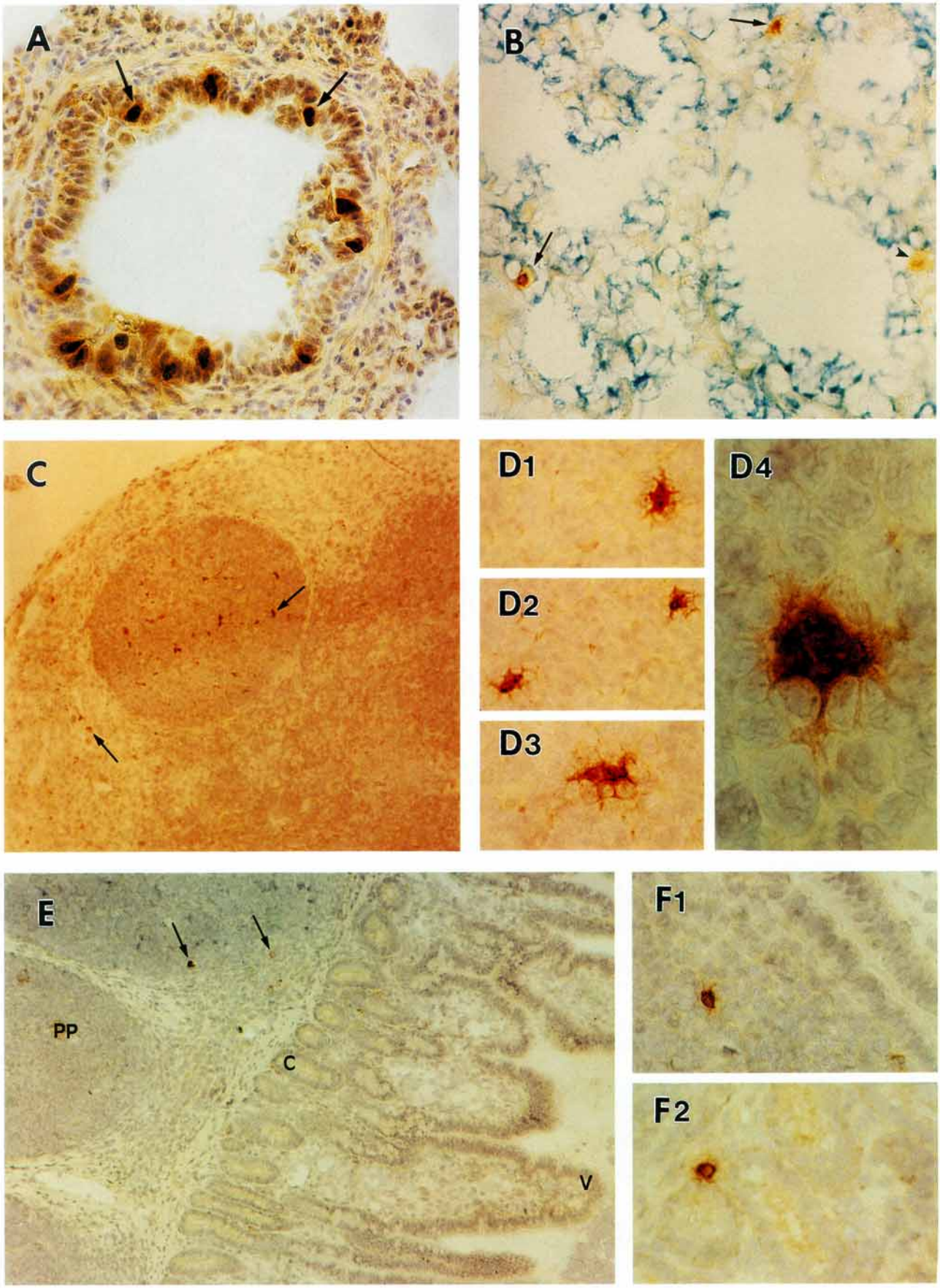
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