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Pseudorabies Virus Glycoprotein gIII Is a Major Target Antigen for Murine and Swine Virus-Specific Cytotoxic T Lymphocytes

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Pseudorabies virus (PrV) is the etiological agent of Aujeszky's disease, a disease that causes heavy economic losses in the swine industry. A rational approach to the generation of an effective vaccine against this virus requires an understanding of the immune response induced by it and of the role of the various viral antigens in inducing such a response. We have constructed mutants of PrV [strain PrV(Ka)] that differ from each other only in expression of the viral nonessential glycoproteins gI, gp63, gX, and gIII (i.e., are otherwise isogenic). These mutants were used to ascertain the importance of each of the nonessential glycoproteins in eliciting a PrV-specific cytotoxic T-lymphocyte (CTL) response in mice and pigs. Immunization of DBA/2 mice and pigs with a thymidine kinase-deficient (TK^-) mutant of PrV elicits the formation of cytotoxic cells that specifically lyse syngeneic infected target cells. These PrV-specific cytolytic cells have the phenotype of major histocompatibility complex class I antigen-restricted CTLs. The relative number of CTLs specific for glycoproteins gI, gp63, gX, and gIII induced in mice vaccinated with a TK^- mutant of PrV was ascertained by comparing their levels of cytotoxicity against syngeneic cells infected with either wild-type virus or $gI^-/gp63^-$, gX^-, or $gIII^-$ virus deletion mutants. The PrV-specific CLTs were significantly less effective in lysing $gIII^-$-infected targets than in lysing $gI^-/gp63^-$, gX^-, or wild-type-infected targets. The in vitro secondary CTL response of lymphocytes obtained from either mice or pigs 6 or more weeks after immunization with a TK^- mutant of PrV was also tested. Lymphocytes obtained from these animals were cultured with different glycoprotein-deficient mutants of PrV, and their cytolytic activities against wild-type-infected targets were ascertained. The importance of each of the nonessential viral glycoproteins in eliciting CTLs was assessed from the effectiveness of each of the virus mutants to stimulate the secondary anti-PrV CTL response. Cultures of both murine or swine lymphocytes that had been stimulated with $gIII^-$ virus contained only approximately half as many lytic units as did those stimulated with either wild-type virus, a gX^- virus mutant, or a $gI^-/gp63^-$ virus mutant. Thus, a large proportion of the PrV-specific CTLs that are induced by immunization with PrV of both mice and pigs are directed against gIII. Furthermore, glycoproteins gI, gp63, and gX play at most a minor role in the CTL response of these animals to PrV.

Pseudorabies virus (PrV; suid herpesvirus 1) is the etiologic agent of Aujeszky's disease, a disease that is lethal to young pigs and that causes important economic losses (8). Because of the significant economic losses caused by this disease, vaccination of pigs with attenuated or killed vaccines is practiced in many countries. However, eradication of the disease has not as yet been achieved. A rational approach to the control of Aujeszky's disease requires an understanding of the immune response against infection with PrV and identification of the viral antigens responsible for eliciting a protective immune response. However, neither of these determinants is as yet well understood.

Evidence that infection with PrV elicits both humoral and cell-mediated immune responses is widely recognized (9, 10, 11, 42, 49). A humoral immune response after experimental infection or vaccination of pigs with PrV has been detected that consists of virus-neutralizing antibodies, antibodies capable of mediating antibody-dependent cell-mediated cytotoxicity, and antibodies capable of mediating complement-mediated lysis of PrV-infected target cells (20, 21, 50, 51). Much of the humoral neutralizing response in naturally and experimentally infected pigs is directed against virus-encoded glycoproteins (44), in particular, against glycoprotein gIII (1). Furthermore, passive immunization of mice or pigs with monoclonal antibodies (MAbs) specific against gp50, gIII, and gII protects them from lethal challenge with virulent virus (15, 45; T. Ben-Porat, unpublished results). However, the level of antibodies present in vaccinated animals does not correlate with protection against challenge with virulent virus (21), indicating that although antibodies can provide some protection, they may not play a pivotal role in protective immunity. It is therefore of interest to elucidate the types of cell-mediated immune responses elicited by infection of animals with PrV. Furthermore, the well-known importance of cell-mediated immunity, in particular of cytotoxic T lymphocytes (CTLs), in the protection against disease caused by other herpesviruses (4, 17, 18, 29) suggests that cell-mediated immune responses may play a similar role in the control of infections with PrV. An understanding of the factors contributing to cell-mediated immunity against PrV infections is therefore crucial to the development of a successful vaccination program.

Some studies dealing with specific cellular immune responses to PrV that may affect control of PrV infections have appeared. It has been established that in response to viral stimuli, PrV-immune swine lymphocytes proliferate and secrete lymphokines (10, 42, 49). An increase in major histocompatibility complex (MHC)-unrestricted cell-mediated cytotoxicity against PrV-infected and uninfected cells

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has also been detected after infection or vaccination of pigs with PrV (50, 51). Furthermore, the presence of cytotoxic lymphocytes specific for cells infected with PrV in the draining lymph nodes of an intranasally infected pig has been reported (19).

Envelope glycoproteins of PrV are known to be important in the induction of protective immunity against challenge with virulent PrV (14, 35). Therefore, an understanding of the role that each of the envelope glycoproteins of PrV plays in cell-mediated immunity should help in the development of effective vaccines against PrV.

The aim of the experiments presented in this report was to study the CTL response of mice and pigs to nonessential envelope glycoproteins of PrV. This is of particular interest because vaccines deficient in these glycoproteins have been and are being developed. We present evidence that a large proportion of the PrV-specific CTLs in mice and in swine is directed against glycoprotein gIII, while a small proportion only (if any) of these CTLs is directed against glycoprotein gI, gp63, or gX.

MATERIALS AND METHODS

Cell cultures, virus mutants, and animals. Rabbit kidney and Madin-Darby bovine kidney cells were cultivated in Eagle synthetic medium containing 5% calf serum. L (*H-2*^{k}) cells expressing the *H-2L*^{d} or H-2D^d molecule (a generous gift from James Forman, University of Texas Southwestern Medical Center at Dallas) were cultured in hypoxanthine-aminopterin-thymidine medium supplemented with 5% fetal bovine serum, glutamine (1 mM), and gentamicin (50 μg/ml). A continuous line of mouse embryo fibroblasts (MESV) and two continuous lines of pig testis cells (PITSV-29 and PITSV-30) were obtained by transforming DBA/2 (*H-2*^{d}) mouse embryo fibroblasts and pig testis cells, respectively (at the second passage), with simian virus 40 L929 (*H-2*^{k}) and PK(15) cells were purchased from The American Tissue Culture Collection. These cells were cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum. Cells of the human erythroleukemia cell line K562, a gift from Jim Forbes (Vanderbilt University), were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% calf serum. Murine lymphocytes were cultured in RPMI supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 10 mM), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and tylosin (1 μg/ml)). Swine peripheral blood lymphocytes were cultured in complete RPMI supplemented as described above except that fetal bovine serum was substituted with CPSR-2 (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 10% (vol/vol) (CPSR-RPMI).

PrV(Ka) is a strain that has been carried in our laboratory for more than 25 years. Isolation and characterization of the mutants defective in the glycoprotein genes encoding gI and gp63, gX, and gIII, used in this study, have been described (26–28, 41). Mutants defective in the expression of thymidine kinase (TK$^-$) were isolated from each as described previously (22).

DBA/2 (*H-2*^{d}) female mice, 8 to 10 weeks old, were obtained from Harlan Farms, Indianapolis, Ind. Specific-pathogen-free Landrace male pigs weighing 15 kg were obtained from a local PrV-free farm. The pigs and mice were housed in confinement and were given nonmedicated food and water ad libitum.

Virus purification. Virus was purified as described previously (2). Briefly, cell monolayers were infected (multiplicity of infection [MOI], 5 PFU per cell) and incubated for 48 to 72 h in Eagle medium supplemented with 5% calf serum. The cells were scraped into the medium, collected, and clarified by centrifugation at 4,000 × *g* for 10 min. The pellet was suspended in 5 ml of the supernatant, sonicated for 2 min, clarified as before, and pooled with the rest of the supernatant. This procedure was repeated twice to release most of the cell-associated virus. The clarified supernatant containing the intracellular and extracellular virus was centrifuged on a TBSA (0.136 M NaCl, 2.6 mM KCl, 0.01 M Tris hydrochloride, 20 mM $MgCl_2$, and 1.8 mM $CaCl_2$ [pH 7.0] [TBS] plus 1% crystalline bovine serum albumin)–30% sucrose cushion at 15,000 rpm for 1 h in a Beckman SW27 rotor. The virus pellet was resuspended gently in 1 ml of TBSA, and the virus was banded in a 15 to 30% sucrose gradient by centrifugation in a Beckman SW27 rotor at 15,000 rpm for 1 h. The virus band was withdrawn by puncturing the side of the tube. The virus was diluted with TBS and sedimented by centrifugation at 15,000 rpm for 1 h in a Beckman SW27 rotor. The virus pellet was suspended in 1 ml of TBS and stored at −70°C until use.

Antibodies. Murine-derived anti-Thy-1.2 antisera and anti-Lyt-2.1 (CD8) MAb as well as LowTox M rabbit complement were purchased from Cedarlane Laboratories (Accurate Chemicals and Scientific Corp., Westbury, N.Y.). MAb GK1.6 (anti-L3T4) (CD4) was kindly provided by Daniel Colley (Vanderbilt University). The MAbs specific for individual PrV envelope glycoproteins have been described previously (12). MAbs 74-12-4 and 76-2-11, specific for the porcine T-lymphocyte markers CD4 (PT4) and CD8 (PT8), respectively, were kindly provided by Joan Lunney.

Immunization of animals and culture of lymphocytes. The primary CTL response of mice against PrV was induced as follows. Mice were injected with 5×10^5 PFU of PrV(Ka) TK$^-$ virus into each footpad. Six days later, lymph node cells were isolated from the popliteal lymph nodes, seeded into 24-well plates (5×10^6 cells per well) in 2 ml of complete RPMI, and cultured for 3 days at 37°C in a 5% CO_2 atmosphere.

To induce a secondary PrV-specific CTL response in vitro, mice were immunized as described above, and cells were isolated from peripheral lymph nodes 6 to 8 weeks after immunization. The cells were cocultured for 5 days in 24-well plates (5×10^6 cells per well) with syngeneic adherent spleen cells (ASC; 10^6 cells per well) that were infected either with wild-type PrV(Ka) or with a mutant of PrV(Ka) defective in the synthesis of the nonessential glycoprotein gI/gp63, gX, or gIII. Unless otherwise stated, the MOI was 50 PFU per cell (approximately 10^3 particles of the wild-type virus and mutants gI$^-$/gp63$^-$ and gX$^-$ per cell and 10^4 particles of mutant gIII$^-$ per cell).

Swine secondary CTL response was induced as follows. Two pigs were immunized intramuscularly with 10^8 PFU of PrV(Ka) TK$^-$ virus. Peripheral blood mononuclear leukocytes (PBML) were isolated from venous blood of the immunized pigs (or from the preimmune pigs) by centrifugation through a cushion of Histopaque (Sigma). The PBML were cultured for 6 days in 24-well tissue culture plates (8×10^6 cells per well in 2 ml of CPSR-RPMI) with purified wild-type PrV or with a mutant deficient in a nonessential glycoprotein.

Preparation of target cells. Monolayers of cells (2×10^6 to 4×10^6 cells) to be used as targets were infected (MOI, 10 PFU per cell) with the appropriate virus mutant. At the

indicated times after infection, the cells were lifted by brief trypsinization, suspended in 1 ml of complete RPMI containing 200 μCi of ^{51}Cr (NEN-Dupont, Research Products, Boston, Mass.), and incubated for 90 min. They were then washed three times and resuspended in complete RPMI at a concentration of 2×10^5 cells per ml.

Cytotoxicity assay. Labeled target cells (10^4) in 50 μl were distributed into V-bottom 96-well microdilution plates. Effector cells were serially diluted (threefold), and 100 μl of each dilution was added in triplicate to wells containing the target cells. The microdilution plates were centrifuged at 200 × *g* for 2 min and incubated for 4 h at 37°C. The amount of ^{51}Cr released was determined, and the percentage of specific ^{51}Cr release was calculated by the formula (counts per minute released with effector cells − counts per minute released without effector cells/counts per minute released by detergent − counts per minute released without effector cells) × 100. The standard deviation of the mean of triplicate cultures was less than 10%, and the spontaneous release (without effector cells) was always less than 25% of the total counts (released by detergent lysis). One lytic unit of effector cells is defined as the number of effector cells necessary to achieve 30% specific cell lysis (5). The number of lytic units per 10^6 cells was determined as follows: 10^6/number of effector cells required to achieve 30% specific lysis × number of target cells in a test well.

Depletion of effector cell subpopulations with antibody and complement. Effector cells (10^7) were suspended in 1 ml of RPMI containing 0.3 mg of bovine serum albumin as well as the specific antibodies. Final concentrations of the antibodies used for murine effectors were 1:10 for anti-Lyt-2.1 and anti-Thy-1.2 and 1:50 for anti-L3T4. Antibodies for porcine effectors, anti-PT4, and anti-PT8 were used at a final concentration of 1:10. After 1 h of incubation at 4°C, the cells were centrifuged and suspended in 1 ml of LowTox M rabbit complement diluted 1:10 in RPMI and further incubated at 37°C for 1 h. The antibody and complement treatments were repeated twice. The cells were washed, counted, and adjusted to equal numbers, and their ability to lyse ^{51}Cr-labeled target cells was assayed as described above.

Antibody- and complement-mediated ^{51}Cr release assay. Virus-infected ^{51}Cr-labeled target cells (10^4 cells per well in a volume of 100 μl in 96-well plates) were incubated in triplicate with MAbs specific for PrV envelope glycoproteins or with a pig anti-PrV antiserum (final concentration, 1:50). An equal volume of a 1:5 dilution of LowTox M rabbit complement (in complete RPMI) was then added, and the samples were further incubated for 30 min at 37°C. The amount of radioactivity released from the cells was measured in a gamma counter. Spontaneous release was ascertained after incubation of the target cells in the presence of complement alone; it never exceeded 25%. The results were expressed as percent specific ^{51}Cr release, calculated as described above.

RESULTS

PrV encodes at least eight glycoproteins (12). The genes encoding six of these envelope glycoproteins, gI, gII, gIII, gp63, gp50, and a secreted glycoprotein, gX, have been mapped and sequenced (25, 31–33, 38, 39, 46). Of these, gI, gIII, gp63, and gX are nonessential for virus growth in cell culture (1, 24, 32, 40, 47) and therefore can be deleted from the virus genome without affecting its viability. We have used different mutants of PrV defective in the synthesis of one (or two) of these nonessential glycoproteins as a tool to study the relative importance of each in eliciting a cell-mediated immune response against PrV.

Kinetics of expression of PrV glycoproteins on the surface of infected cells. To determine whether the nonessential glycoproteins are expressed on the surface of infected cells, as well as to ascertain the optimal time after infection at which the cells can be used as targets for PrV-reactive CTLs, the kinetics of expression of the glycoproteins on the surface of cells infected with wild-type PrV or with a glycoprotein-defective mutant was ascertained in an antibody- and complement-mediated ^{51}Cr release assay. MESV cells were infected (moi of 10 PFU per cell) with wild-type PrV(Ka) (Fig. 1A), a $gI^-/gp63^-$ virus mutant (Fig. 1B), or a $gIII^-$ virus mutant (Fig. 1C) and labeled with ^{51}Cr. At different times after infection, their susceptibilities to antibody- and complement-mediated ^{51}Cr release were determined as described in Materials and Methods.

The anti-PrV serum and the MAbs against glycoproteins gI and gIII were able to mediate the complement-dependent lysis of wild-type PrV(Ka)-infected cells (but not uninfected cells [data not shown]), indicating that glycoproteins gI and gIII were expressed on the surface of the infected cells. As expected, cells infected with the $gI^-/gp63^-$ mutant virus were lysed only by the anti-PrV serum and anti-gIII MAb, not by the anti-gI MAb, and cells infected with the $gIII^-$ mutant virus were lysed by only the anti-PrV serum and anti-gI MAb, not by the anti-gIII MAb. Interestingly, whereas cells infected with $gIII^-$ were lysed by the anti-PrV serum and the anti-gI MAb with equal efficiency, the anti-PrV serum was more effective than the anti-gI MAb in lysing the wild-type-infected cells. It is possible that this difference is due to the significant proportion of gIII-specific antibodies present in the sera of PrV-infected convalescent pigs (1).

The infected cells also reacted with MAbs against gp50 and gII, but MAbs against gp63 and the secreted glycoprotein gX failed to lyse these cells (data not shown). Whether this is because the MAbs against the latter two glycoproteins failed to interact with these glycoproteins on the cell surface (they do immunoprecipitate these glycoproteins) or whether these glycoproteins are not exposed on the cell surface remains to be ascertained.

Cells infected with wild-type virus or with mutant $gI^-/gp63^-$ (and mutant gX^- [data not shown]) were lysed significantly by 6 h postinfection. The $gIII^-$-infected cells, however, showed a similar degree of lysis only at 12 h postinfection. (The delay in the infectious cycle of $gIII^-$-infected cells is related, at least in part, to the alternative [slower] mode of adsorption and of penetration into the cells this mutant uses [41, 53].) Because of the delay in susceptibility to lysis of $gIII^-$-infected cells, these cells were used as targets at 12 or 14 h postinfection, whereas wild-type-, $gI^-/gp63^-$, and gX^--infected cells were used as targets at 6 or 8 h postinfection, at which times all cells showed similar degrees of susceptibility to antibody- and complement-mediated cell lysis.

Generation of PrV-specific CTLs in mice. To generate a PrV-specific cell-mediated immune response, DBA/2 mice were injected in the footpads with a TK^- mutant of PrV(Ka). Lymphocytes were isolated from the draining popliteal lymph node 6 days after injection and cultured for 3 days in the presence of either PrV-infected or uninfected, irradiated syngeneic splenocytes.

Lymphocytes isolated from PrV-infected animals were able to lyse PrV-infected (but not uninfected) syngeneic target cells whether or not they had been stimulated with infected spleen cells; lymphocytes isolated from naive ani-

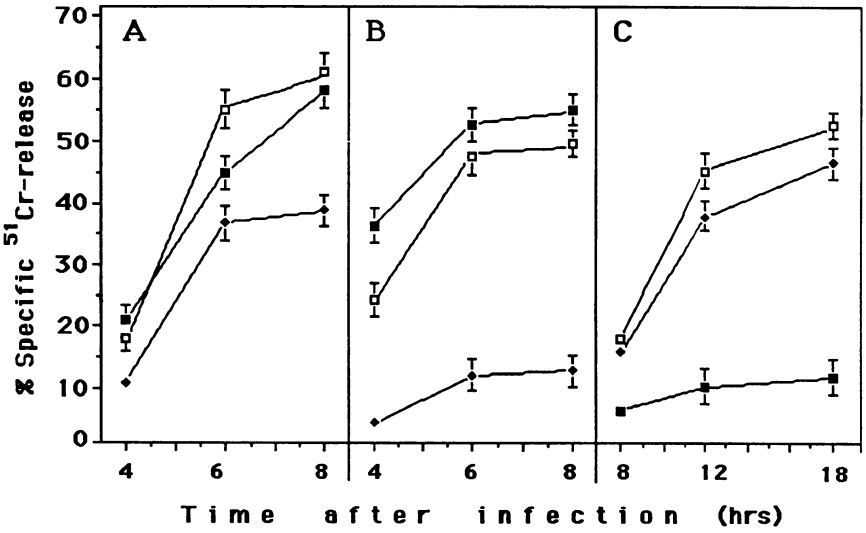

FIG. 1. Kinetics of expression of PrV glycoproteins on the surface of infected cells. MESV cells were infected (MOI, 10 PFU per cell) with PrV(Ka) (A), mutant gI$^-$ (B), or mutant gIII$^-$ (C). They were labeled with ^{51}Cr and reacted at the indicated times for 1 h with anti-PrV serum (□), anti-gI MAb (◆), or anti-gIII MAb (■), followed by incubation for 1 h with rabbit complement. The percent specific ^{51}Cr release was ascertained. Background release at the end of the experiment was <25%.

mals and cultured with PrV-infected spleen cells did not (Table 1). Thus, a PrV-specific lytic activity was generated in the PrV-immunized animals which, under the experimental conditions used, did not require additional antigenic stimulation during culture in vitro, probably because of the presence of residual viral antigen in the lymphocyte culture. (Since the presence of live virus in the culture did not affect lysis, it appears that the effector cells are not susceptible to PrV infection.) In subsequent experiments, the lymphocytes were therefore not exposed to antigenic stimulation during culture in vitro before assay.

The lymphocytes isolated from the PrV-infected DBA/2 mice lysed PrV-infected syngeneic MESV (*H-2^d*) cells but not allogeneic L929 (*H-2^k*) cells (Fig. 2). They are therefore MHC restricted. Furthermore, the cytotoxic cells lysed PrV-infected but not uninfected L cells expressing the *H-2L^d* but not the *H-2D^d* molecule (Fig. 2). Thus, these cytotoxic cells were restricted by the *H-2L^d* but not by the *H-2D^d* molecule.

To further define the phenotype of the cells that mediate the lysis of the PrV-infected target cells, the effector cells were treated with complement and different antibodies against T-cell-specific cell surface antigens. Their lytic activity against PrV-infected syngeneic MESV cells was ascertained thereafter. Treatment of the effector cells with antibodies against the T-cell antigen Thy-1 or against the CTL phenotypic marker CD8 (Lyt-2) plus complement eliminated 70% of the lytic activity against PrV-infected cells (Fig. 3). Treatment with antibodies against the helper T-cell phenotypic marker CD4 (L3T4) and complement, on the other hand, did not decrease the lytic activity of the effector cells. Thus, the PrV-specific cytotoxic cells have the cell surface phenotype of classical class I MHC-restricted CTLs, Thy-1$^+$ L3T4$^-$ Lyt-2$^+$ (43).

Role of PrV glycoproteins in the PrV-specific CTL response of mice. To ascertain the role that each of the nonessential PrV glycoproteins plays as a target antigen for PrV-specific CTLs, we compared the ability of the anti-PrV CTLs to lyse syngeneic cells infected with wild-type PrV or with mutants of this virus that do not express either glycoprotein gI and gp63 (gI$^-$/gp63$^-$), gX (gX$^-$), or gIII (gIII$^-$). Using this approach, we consistently observed that at the same effector/target ratios, the percent specific lysis of cells infected with mutant gI$^-$/gp63$^-$ or gX$^-$ was the same as that of cells infected with wild-type virus (Fig. 4A). On the other hand, the effector/target ratio needed to obtain comparable lysis of gIII$^-$-infected cells was significantly higher. Effector/target ratios required to lyse 30% of the target cells varied some-

TABLE 1. Generation of PrV-specific murine CTLs[a]

Effector cell population	Antigenic stimulus	% Specific lysis ± SE of syngeneic cells[b]					
		PrV infected			Mock infected		
		100:1	50:1	25:1	100:1	50:1	25:1
Immune	PrV-infected splenocytes	42 ± 3	28 ± 2	17 ± 3	12 ± 2	7 ± 3	5 ± 2
	Uninfected splenocytes	38 ± 2	26 ± 4	14 ± 2	9 ± 2	7 ± 2	5 ± 2
Naive	PrV-infected splenocytes	10 ± 2	7 ± 3	9 ± 3	7 ± 3	5 ± 2	5 ± 2

[a] Popliteal lymph node cells were isolated from uninfected DBA/2 mice or from mice 6 days after footpad inoculation with 10^6 PFU of PrV TK$^-$. They were incubated (5×10^6 cells per well) for 3 days with either 5×10^6 PrV-infected or uninfected irradiated (3,000 R) splenocytes from DBA/2 naive mice as a source of antigen. Cytolytic activity against ^{51}Cr-labeled infected or mock-infected mouse embryo fibroblasts at the indicated effector/target ratios was determined.

[b] Primary cultures of DBA/2 mouse embryo fibroblasts infected with PrV (MOI, 10 PFU per cell) or mock infected were labeled with ^{51}Cr and used as targets 6 h postinfection in a standard 4-h ^{51}Cr release assay.

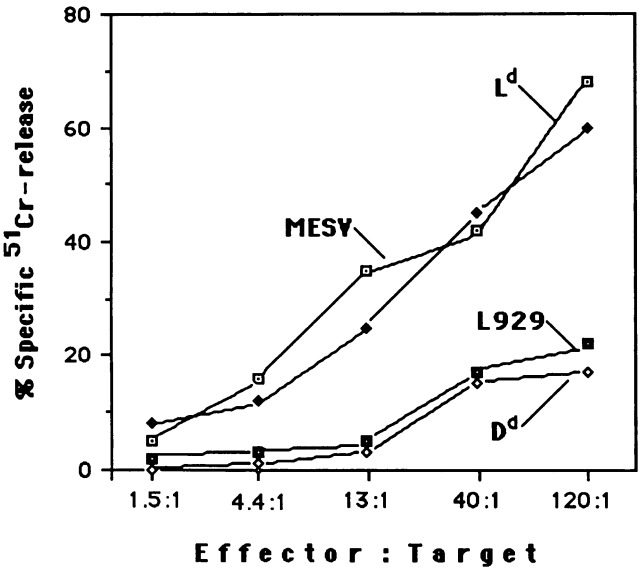

FIG. 2. MHC restriction of PrV-specific cytolytic cells. PrV-immune cells obtained from DBA/2 (*H-2*^{d}) mice as described in Materials and Methods were tested for cytolytic activity against the ^{51}Cr-labeled target cells infected with PrV (MOI, 10 PFU per cell). Cells tested: MESV (*H-2*^{d}), L929 (*H-2*^{k}), D^d (L cells [*H-2*^{k}] expressing the *H-2D*^{d} molecule), and L^d (L cells expressing the *H-2L*^{d} molecule).

what in different experiments. In four different experiments, they were 50:1, 32:1, 21:1, and 15:1 for gIII$^-$-infected cells and 10:1, 5:1, 12:1, and 7:1 for wild-type-infected cells. Thus, two- to sixfold-more effector cells appeared to be required to achieve 30% specific lysis of gIII$^-$-infected cells than of wild-type-infected cells.

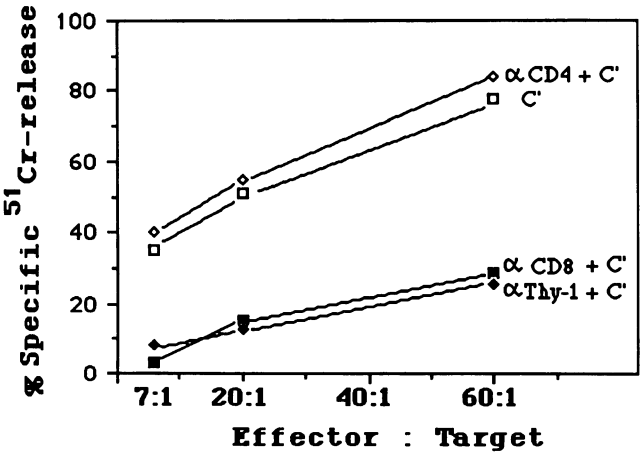

FIG. 3. Effect of anti-Thy, anti-CD4, and anti-CD8 antibodies on murine anti-PrV cytolytic effector cells. Popliteal lymph node cells isolated from DBA/2 mice 6 days after footpad inoculation with PrV TK$^-$ were cultured in vitro for 3 days and then treated with either Thy-1-, CD4-, or CD8-specific antibodies and complement. The lytic activity of the surviving cells against PrV-infected syngeneic MESV cells was then determined in a 4-h ^{51}Cr release assay. Lysis at a 60:1 effector/target ratio of syngeneic mock-infected MESV cells was 12% and that against allogeneic PrV-infected L929 cells was 13% (data not shown), indicating the specificity and MHC restriction of the effector cell population.

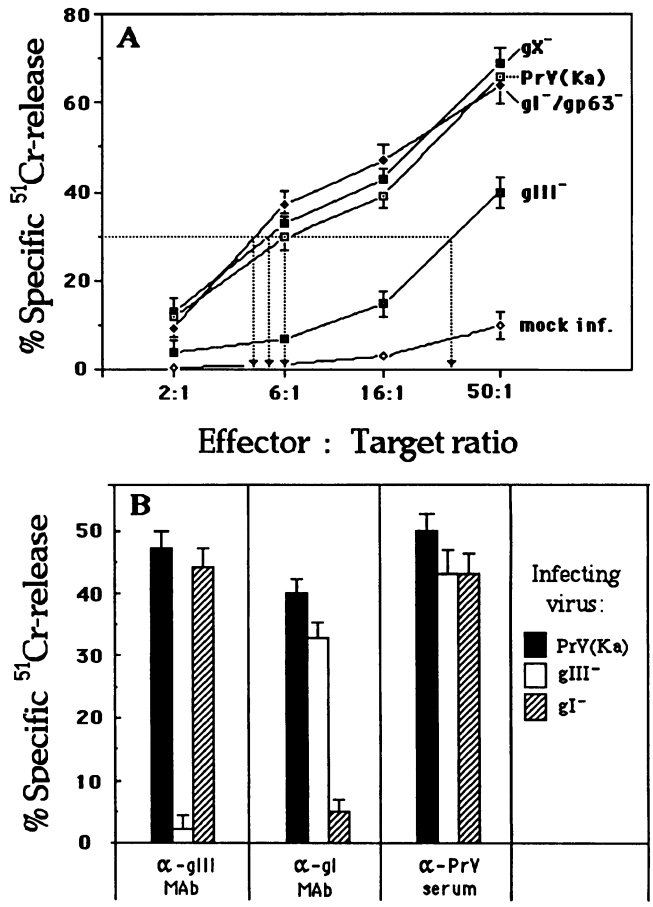

FIG. 4. PrV nonessential glycoproteins as target antigens for PrV-specific CTLs. (A) PrV-immune cells obtained from DBA/2 mice as described in Materials and Methods were tested for cytolytic activity against ^{51}Cr-labeled uninfected MESV cells or MESV cells infected with wild-type PrV(Ka) or with mutant gI$^-$/gp63$^-$, gIII$^-$, or gX$^-$ in a 4-h ^{51}Cr release assay. (B) The target cells used in the experiment shown in panel A were incubated for 1 h with anti-PrV antiserum or with an MAb against glycoprotein gI or gIII. They were then incubated for 1 h with rabbit complement, and percent specific lysis was determined.

To rule out the possibility that the higher number of effector cells required to achieve comparable lysis of gIII$^-$-infected cells than of wild-type-infected cells is due to the lower susceptibility of the former to CTL-mediated lysis (because of a low level of expression of surface glycoproteins in gIII$^-$-infected cells or because of the low intrinsic fragility of these cells), we simultaneously measured the susceptibility of the same target cells to anti-PrV antibody- and complement-mediated lysis. Lysis of all of the target cells by PrV-specific antiserum and complement was similar (Fig. 4B). Thus, the target cells infected with mutant gIII$^-$ did not appear to be less susceptible to lysis than were the target cells infected with the other mutants or with wild-type virus.

Since gI$^-$/gp63$^-$- and gX$^-$-infected cells were as sensitive to CTL-mediated lysis as were wild-type-infected cells, our results would indicate that the PrV-specific CTLs against PrV-infected target cells are not directed against glycoproteins gI, gp63, and gX. On the other hand, because the gIII$^-$-infected targets were less susceptible to CTL-mediated lysis, it would appear that a significant proportion of the

PrV-reactive CTLs may be directed against glycoprotein gIII. However, despite the fact that gIII$^-$-infected targets were as sensitive to antibody- and complement-mediated cell lysis as were targets infected with wild-type virus or with mutant gI$^-$, we felt it necessary to further eliminate the possibility that a differential susceptibility of the target cells to lysis contributed to our results. We therefore used a different approach and tested the abilities of wild-type- and mutant-infected cells to stimulate in vitro a secondary CTL response against PrV-infected cells. The rationale behind this approach was that if a significant proportion of the PrV-specific memory CTLs is directed against a particular glycoprotein, the absence of this glycoprotein during induction of the secondary CTL response should generate a significantly lower number of lytic units. Since in this type of experiment the lytic activity of the different CTL populations is tested against the same target cells, possible differences in susceptibility of the target cells to lysis can be eliminated as a source of experimental error.

To obtain lymph node cell populations that would respond to secondary antigenic stimulation in vitro, peripheral lymph nodes were obtained from DBA/2 mice 6 to 8 weeks after they had been inoculated with PrV TK$^-$. The isolation of lymph node cells was delayed until close to 2 months after inoculation because by this time CTL activity was not detectable unless the cells were restimulated with viral antigen during cultivation in vitro. The lymph node cells were cocultured with irradiated (2,500 R) syngeneic ASC that had been mock infected, infected with wild-type PrV, or infected with mutant gI$^-$/gp63$^-$, gIII$^-$, or gX$^-$. The cells were cultured for 5 days, and their lytic activity against syngeneic PrV(Ka)-infected MESV cells was tested in a 4-h ^{51}Cr release assay (Fig. 5).

As expected, only low levels of lytic activity were detected in lymphocyte cultures stimulated with mock-infected ASC, whereas lymphocyte cultures stimulated with any one of the PrV-infected ASC had substantial lytic activity against the PrV-infected MESV cells (Fig. 5). The number of effector cells required to achieve 30% lysis of the target cells was similar when the lymphocyte cultures had been stimulated with ASC that had been infected with wild-type virus or with the double-deletion mutant gI$^-$/gp63$^-$. However, three times as many effector cells were required to achieve 30% specific lysis of the same PrV-infected target cells when the lymphocyte cultures had been cocultured with gIII$^-$-infected ASC. Similar results were obtained in several different experiments (Table 2). In all cases, lymphocyte cultures stimulated with gIII$^-$-infected ASC generated a lower number of lytic units against PrV(Ka)-infected MESV cells than did cultures stimulated with ASC infected with either wild-type virus, gI$^-$/gp63$^-$, or gX$^-$. Significant lytic activity against mock-infected MESV cells or against PrV-(Ka)-infected allogeneic L cells was not detected in any of the experiments (Table 2).

To ensure that the lower ability of the gIII$^-$-infected ACS than of the wild-type-infected ACS to stimulate an anti-PrV lytic activity was not related to lower doses of mutant gIII$^-$ than of wild-type virus with which the stimulator cells were infected (gIII$^-$ virus does not adsorb to its host cells as effectively as does gIII$^+$ virus [41]), the effect of adding increasing amounts of virus to the stimulator ASC-lymphocyte culture was ascertained. The lytic activity observed when the ASC-lymphocyte cultures were stimulated with mutant gIII$^-$ was lower than that obtained when the ASC-lymphocyte cultures were stimulated with wild-type virus over a large range of doses (Fig. 6). Furthermore, the peak

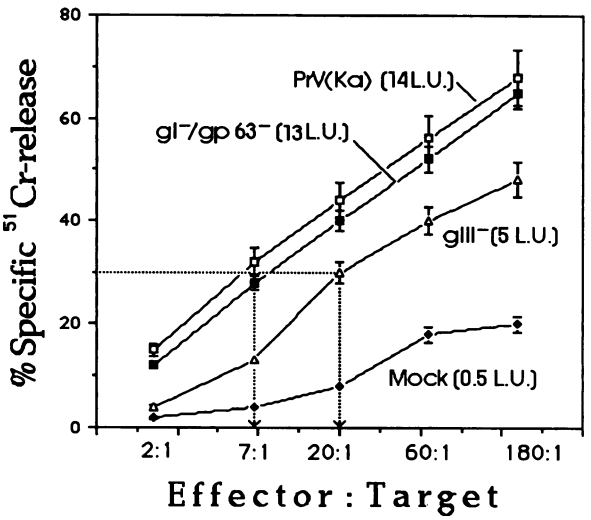

FIG. 5. Ability of cells infected with different mutants of PrV defective in the expression of nonessential glycoproteins to elicit a murine anti-PrV secondary CTL response. Lymph node cells (5 × 10^6 per well) isolated from DBA/2 mice 6 weeks after footpad immunization with PrV TK$^-$ were cocultured for 5 days with irradiated syngeneic ASC (10^6 per well) that had been mock infected, infected with wild-type PrV(Ka), or infected with mutant gI$^-$/gp63$^-$ or gIII$^-$. The lytic activity of the effector cells was tested against ^{51}Cr-labeled PrV-infected MESV cells in a 4-h ^{51}Cr release assay. Lysis at an effector/target ratio of 100:1 of syngeneic mock-infected MESV cells was 10% and of allogeneic PrV-infected L929 cells was 9%, indicating the specificity and MHC restriction of the effector cell population. One lytic unit is the number of effector cells required to achieve 30% specific lysis. The number of lytic units in 10^6 cells was determined as described in the text.

lysis occurred at similar doses for both. These results indicate that the lower induction of lytic activity by the gIII$^-$-infected stimulator cells was not related to the virus (antigen) dose. We conclude that the lower lytic activity of the lymphocyte cultures stimulated by mutant gIII$^-$ reflects the fact that a significant proportion of the secondary PrV-specific CTL response in DBA/2 mice is directed against glycoprotein gIII, i.e., that gIII is a major target antigen for PrV-specific CTLs. Glycoproteins gI, gp63, and gX, on the other hand, play at most a minor role in the reactivity of these cells.

Induction of porcine PrV-specific CTLs. Since swine are the natural host of PrV, it was of interest to determine whether results similar to those obtained with DBA/2 mice would also be obtained with swine. To test the MHC-restricted lytic activity of swine CTLs against PrV, autologous cell lines from two prospective donors of PrV-immune lymphocytes were established by transforming second-passage testis cells with simian virus 40 (PITSV cells). PBML isolated from these animals before immunization were infected with purified wild-type PrV(Ka) and cultured for 6 days, and their ability to lyse autologous PrV-infected or mock-infected cells was tested in a 4-h ^{51}Cr release assay. The castrated pigs were then vaccinated by intramuscular injection of purified PrV(Ka) TK$^-$. Four weeks after immunization and at intervals thereafter, the ability of cultured PBML obtained from the immunized animals to lyse PrV-infected cells was ascertained as had been done before immunization.

Stimulation with purified PrV of PBML obtained from the

TABLE 2. Stimulation of a secondary CTL response by PrV mutants defective in the expression of nonessential glycoproteins[a]

Expt	Antigenic stimulus	Lytic activity (lytic units/10^6 cells) against target cells[b]		
		MESV		L929 [PrV(Ka) infected]
		PrV(Ka) infected	Mock infected	
1	PrV(Ka)	12.0	<0.1	<0.9
	gIII$^-$	6.0	<0.1	<0.9
	None	0.1	<0.1	<0.1
2	PrV(Ka)	10.0	<0.2	<0.1
	gIII$^-$	4.0	<0.2	<0.1
	None	0.4	<0.1	<0.1
3	PrV(Ka)	12.5	<0.1	<0.1
	gIII$^-$	5.0	<0.1	<0.1
	None	0.3	<0.1	<0.1
4	PrV(Ka)	14.0	<0.1	<0.1
	gI$^-$/gp63$^-$	15.0	<0.1	<0.1
	None	0.7	<0.1	<0.1
5	PrV(Ka)	5.0	<0.1	<0.1
	gX$^-$	5.0	<0.1	<0.1
	gIII$^-$	1.7	<0.1	<0.1
	None	0.7	<0.1	<0.1

[a] Lymph node cells (5×10^6 per well) isolated from DBA/2 mice 6 weeks after footpad immunization with PrV TK$^-$ were cocultured for 5 days with irradiated (2,500 R) syngeneic ASC (10^6 per well) that had been either mock infected, infected with wild-type PrV(Ka), or infected with mutant gI$^-$/gp63$^-$, gIII$^-$, or gX$^-$. The cultured cells were tested for cytolytic activity against the indicated ^{51}Cr-labeled target cells infected with PrV(Ka) (MOI, 10 PFU per cell) or uninfected target cells.

[b] Determined as described in the text.

TABLE 3. Generation of PrV-specific swine CTLs[a]

Effector cells[a]	Antigenic stimulus[b]	% Specific lysis ± SE of autologous cells[c]			
		PrV infected		Mock infected	
		100:1	50:1	100:1	50:1
Naive					
Pig 30	−	10 ± 0.9	8 ± 0.6	13 ± 1.1	10 ± 0.9
	+	11 ± 1.2	9 ± 0.8	12 ± 1.2	11 ± 1.1
Pig 29	−	12 ± 1.8	4 ± 1.1	10 ± 0.9	6 ± 0.7
	+	15 ± 2.2	5 ± 1.2	13 ± 1.8	8 ± 1.1
Immune					
Pig 30	−	15 ± 2.1	6 ± 0.7	10 ± 0.9	5 ± 0.4
	+	60 ± 6.2	47 ± 4.3	25 ± 2.5	17 ± 2.1
Pig 29	−	11 ± 1.6	7 ± 0.9	10 ± 1.5	8 ± 0.9
	+	67 ± 6.3	33 ± 4.7	20 ± 2.6	12 ± 1.3

[a] PBML were isolated from venous blood of pigs by centrifugation through a cushion of Hystopaque. The cells (8×10^6 in 2 ml per well) were cultured for 6 days with or without virus as antigenic stimulus and tested for lytic activity against the indicated target cells in a 4-h ^{51}Cr release assay.

[b] Purified PrV(Ka) was added on day 0 to the culture wells at a final concentration of 2×10^5 PFU per well.

[c] PrV-infected or mock-infected autologous PITSV cells (PITSV-29 for pig 29 and PITSV-30 for pig 30) were ^{51}Cr labeled and used as targets at 6 h postinfection. Values at the indicated effector/target ratios were calculated as described in Materials and Methods.

PrV-immune animals resulted in the generation of effector cells capable of lysing PrV-infected but not mock-infected autologous cells. (Since the lytic activity of the PBML obtained from the vaccinated pigs was stimulated by the addition of live PrV, it is clear that the effector cells are not susceptible to infection with PrV.) When the PrV-immune PBML were cultured in the absence of viral antigen, only background levels of lytic activity were generated (Table 3). Stimulation with PrV of PBML isolated from the preimmune animals did not generate cells capable of lysing PrV-infected or mock-infected autologous cells (Table 3). Thus, cytolytic cells against PrV-infected autologous cells were generated only when the PBML obtained from animals that had been vaccinated with PrV were stimulated in vitro with PrV. Since only background levels of lytic activity were detected against allogeneic cells infected with PrV (Fig. 7), the lytic activity of the effector cells was restricted by MHC antigens. Furthermore, only background levels of lytic activity were detected against the natural killer (NK) target cell line K562 (Fig. 7), suggesting that the cytolytic cells were not NKs but may have been CTLs.

Phenotype of the swine effector cells. The phenotype of the

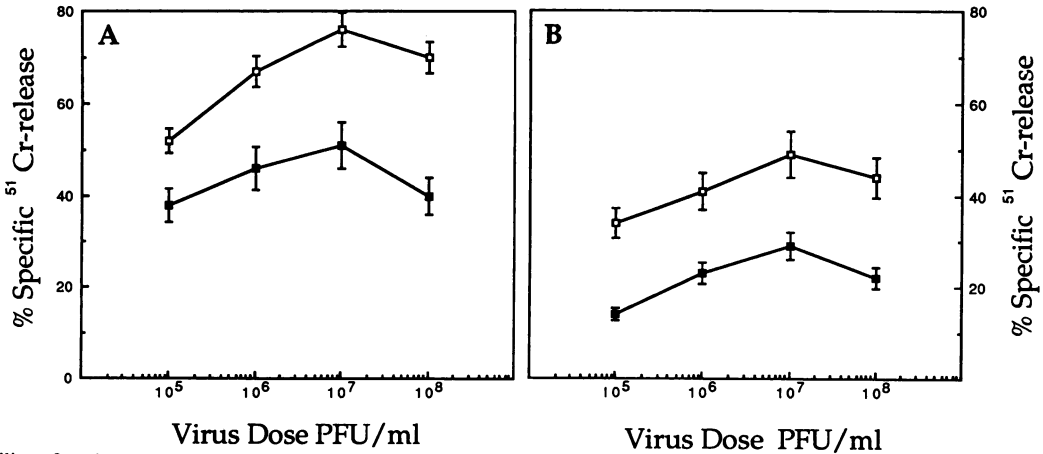

FIG. 6. Ability of various doses of wild-type PrV(Ka) or of a virus mutant defective in the expression of gIII to stimulate a murine anti-PrV secondary CTL response. Peripheral lymph node cells isolated from DBA/2 mice 6 weeks after footpad immunization with PrV(Ka) TK$^-$ were cultured as described in the legend to Fig. 5 with increasing concentrations of wild-type PrV (□) or of mutant gIII$^-$ (■). After 5 days in culture, the effector cells were tested for lytic activity against syngeneic PrV-infected MESV cells in a 4-h ^{51}Cr release assay. Effector/target ratios were 100:1 (A) and 33:1 (B). Lysis of mock-infected MESV cells or PrV-infected allogeneic L cells (*H-2*^{k}) at a 100:1 ratio was <15%.

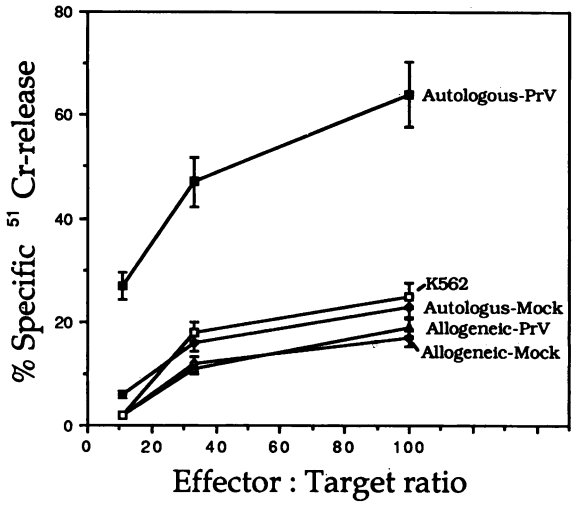

FIG. 7. Restriction of porcine anti-PrV cytolytic activity by the genetic compatibility between effector and target cells. Effector cells specific against PrV were generated from PBML isolated from pig 30 as described in footnote *a* of Table 3 and tested in a 4-h ^{51}Cr release assay against the indicated target cells. Autologous cells were PITSV-30; allogenic cells were PK cells.

cells responsible for the lytic activity against autologous PrV-infected cells in the PBML population obtained from one of the immunized pigs (pig 30; pig 29 died of causes unrelated to infection with PrV) was ascertained (Fig. 8). To this end, we determined the ability of antibodies against pig T-cell surface antigens PT4 (CD4) and PT8 (CD8) to deplete the lytic activity in the presence of complement (30). PBML isolated from the immune animal were cultured for 6 days with purified PrV and treated with complement alone, with anti-PT4 antibody and complement, or with anti-PT8 antibody and complement. The number of viable cells that remained was determined, and their lytic activity against PrV-infected autologous cells was ascertained. Treatment of the effector cells with anti-PT4 serum and complement did not decrease their lytic activity, but treatment with anti-PT8 serum and complement eliminated over 60% of this activity (Fig. 8). Thus, the secondary anti-PrV cytotoxic cell response that was induced by PrV in PBML obtained from PrV-immune pigs was mediated by cells with the phenotype of porcine CTL, $PT4^-$ $PT8^+$ (30).

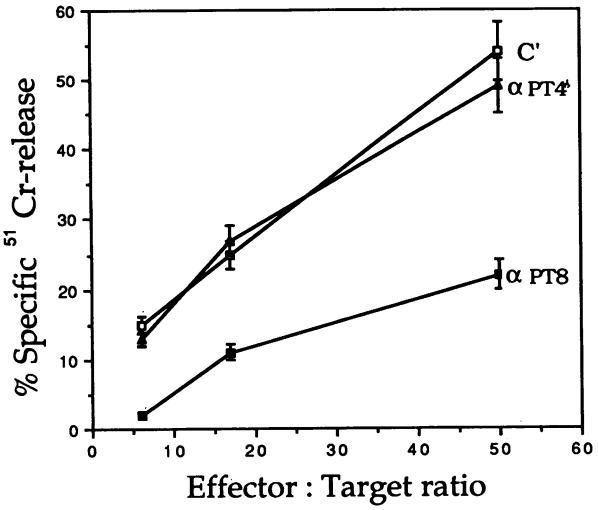

FIG. 8. Effect of anti-PT4 and anti-PT8 antibodies and complement on porcine anti-PrV cytolytic effector cells. Anti-PrV cytolytic cells generated as described in footnote *a* of Table 3 were treated with complement alone or with an antibody specific against PT4 or PT8 and complement. The surviving cells were counted, and equal numbers of viable cells were tested for lytic activity against PrV-infected autologous cells. Lysis of mock-infected autologous cells at a 50:1 effector/target ratio was <15% in all cases.

Role of the nonessential PrV envelope glycoprotein in porcine PrV-specific CTL response. To ascertain the role (if any) of the nonessential PrV envelope glycoproteins in the reactivity of porcine PrV-specific CTLs, the ability of purified populations of virus, each lacking a specific nonessential glycoprotein, to stimulate a secondary anti-PrV CTL response of porcine PBML was ascertained (Fig. 9). Stimulation of cultures of PrV-immune pig PBML with either mutant $gI^-/gp63^-$ or mutant gX^- resulted in the generation of as many lytic units against PrV-infected autologous cells as did stimulation with wild-type virus. However, PBML cultures stimulated with purified $gIII^-$ contained only approximately half as many lytic units as did PBML cultures stimulated with wild-type virus (Fig. 9). The induction of the lower number of lytic units by mutant $gIII^-$ than by wild-type virus was not due to a lower dose of $gIII^-$ antigen with which the PBML was inoculated. This was demonstrated by an experiment in which increasing amounts of wild-type virus and mutant $gIII^-$ were added to the PBML cultures to induce the secondary CTL responses (Fig. 10). The optimal stimulating dose of $gIII^-$ resulted in the generation of approximately half as many lytic units as did the optimal stimulating dose of wild-type virus. We conclude that a significant proportion of the secondary PrV-specific CTL response in the particular pig that we have studied is directed against glycoprotein gIII, i.e., that in this animal, gIII was a major target antigen for the CTLs. CTLs against glycoprotein gI, gp63, or gX, on the other hand, appeared to have only a minor role in target cell lysis.

DISCUSSION

This report deals with the characterization of the cellular immune response to infection with PrV and with the relative importance of the nonessential glycoproteins of this virus in this process. The cellular immune responses in two animal species, mice and swine, were investigated. The salient findings of these experiments can be summarized as follows: (i) in both DBA/2 mice and swine, infection with PrV resulted in the appearance of CTLs specific against PrV proteins; and (ii) in both species, a large proportion of these CTLs was directed against glycoprotein gIII, and a specific CTL response against glycoproteins gI, gp63, and gX was not detectable by the methods used.

The PrV-specific CTL response that was induced in both species was restricted by the genetic compatibility of the effector and target cells and was shown to consist of $Thy\text{-}1^+$ $CD8^+$ $CD4^-$ cells in mice and $PT4^-$ $PT8^+$ cells in pigs. Moreover, the murine anti-PrV CTLs were restricted by the *H-2L*^{d} but not by the *H-2D*^{d} class I molecule.

A primary anti-PrV CTL response could be detected in mice by 6 days after inoculation, at which time no antigen restimulation was needed during the culture period in vitro to detect CTL activity against PrV-infected cells. This is probably due to the presence of residual antigen in the draining lymph node. Secondary responses requiring antigen

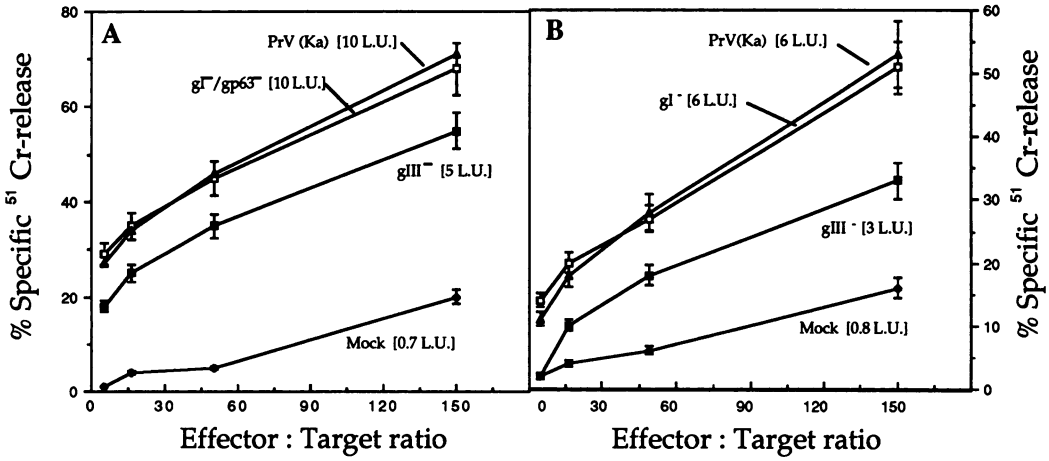

FIG. 9. Ability of mutants of PrV defective in the expression of a nonessential glycoprotein to elicit a porcine anti-PrV secondary CTL response. PBML from PrV-immune pig 30 were cultured as described in footnote *a* of Table 3 with wild-type PrV(Ka) or with the indicated glycoprotein deletion mutant virus. The lytic activity of the different PBML cultures against autologous PrV-infected PITSV-30 cells was tested in a 4-h ^{51}Cr-release assay. Lysis of autologous mock-infected PITSV-30 cells was <15%. Panels A and B show the results of two different experiments.

stimulation during the in vitro culture period could be detected in mice for several weeks after inoculation. Our results therefore show that PrV, like many other viruses, induces a CTL response in mice.

Anti-PrV CTL could also be detected in PBML cultured with purified PrV (devoid of any detectable contaminating foreign proteins) that had been isolated from venous blood of PrV-immune pigs. Since the pathway of lymphocyte recirculation in the pig occurs mainly through the venous blood rather than through the efferent lymph as is the case in other species (3), memory CTLs should be present in peripheral venous blood in significant numbers. We show here that they indeed are; the lytic activity could be detected at the earliest time postimmunization tested (4 weeks) and up to 6 months thereafter (the latest time tested).

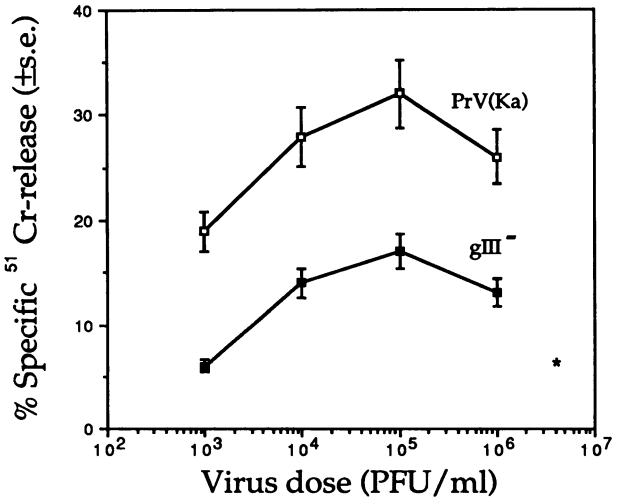

FIG. 10. Ability of various doses of wild-type PrV(Ka) or of mutant gIII$^-$ to stimulate a porcine anti-PrV secondary CTL response. PBML from PrV-immune pig 30 were cultured as described in footnote *a* of Table 3 with the indicated dose of PrV(Ka) (□) or mutant gIII$^-$ (■). The lytic activity against autologous (PITSV-30) PrV-infected cells was tested in a 4-h ^{51}Cr release assay. *, Level of lytic activity of PBML cultures that had not been stimulated with viral antigen.

The porcine anti-PrV cytolytic effector cells that we have detected have the same phenotype as do the porcine alloreactive CTLs detected by Pescovitz et al. (30); i.e., they are PT4$^-$ PT8$^+$. That these cells are true CTLs is indicated by the following: (a) the effector cells were able to lyse PrV-infected but not mock-infected autologous cells, (b) no cytolytic activity was detected when the PrV-immune PBML were cultured in the absence of PrV antigenic stimulus, (c) PrV-infected or uninfected allogeneic cells were not lysed by the PBML that had been stimulated with PrV, (d) the cytolytic activity was detected only when PrV-immune cells but not preimmune cells were stimulated in vitro with viral antigen, and (e) the cytolytic activity of the cultures was depleted by treatment with anti-PT8, indicating that the cytolytic cells are probably classical CTLs. Furthermore, it is unlikely that the cytolytic activity observed against PrV-infected autologous cells was mediated by NK cells because K562 cells, which have been shown to be susceptible to porcine NK lytic activity (34), were not lysed. These observations argue strongly that most of the lytic activity against autologous PrV-infected cells of the porcine anti-PrV cytolytic cells was mediated by porcine CTLs. However, we cannot rule out the possibility that a small percentage of the lytic activity that we have detected is mediated by NK cells. To our knowledge, this is the first report in which virus-specific cytotoxic T cells have been demonstrated in the pig.

The role of nonessential viral envelope glycoproteins in eliciting CTLs was assessed by using otherwise isogenic mutants of PrV that differ only in that each lacks a specific glycoprotein. In the murine system, we first compared the lytic activity of primary PrV-immune lymphocytes against cells infected with wild-type PrV or with one of three glycoprotein deletion mutants (gI$^-$/gp63$^-$, gX$^-$, or gIII$^-$). Using this method, we observed that at the same effector/target ratios, the percent specific lysis of gIII$^-$-infected target cells was consistently lower than that of wild-type PrV, gI$^-$/gp63$^-$, or gX$^-$-infected target cells. These observations indicated either that a significant proportion of the PrV-specific CTLs were directed against gIII or that cells

infected with mutant gIII$^-$ were less susceptible to lysis than were cells infected with the other virus mutants (although the different target cells appeared to have similar susceptibilities to antiviral specific lytic immune mechanisms, as detected by antibody- and complement-mediated lysis).

To ensure that the lower effector/target ratio required to lyse wild-type-infected cells than to lyse gIII$^-$-infected cells was not due to a difference in the susceptibility of the target cells, we used an alternative approach. In these experiments, memory CTLs isolated from PrV-immune mice several weeks after immunization were stimulated in vitro with syngeneic ASC infected with wild-type PrV or with one of the three glycoprotein deletion mutants. The lytic activities of the different effector cells were then compared by using in all cases the same wild-type-infected target cells. The results obtained showed that the number of lytic units generated when the effector cells were stimulated with gIII$^-$-infected cells was smaller than that obtained after stimulation with wild-type-, gI$^-$/gp63$^-$-, or gX$^-$-infected cells. The lower lytic activity obtained with gIII$^-$-infected stimulator cells held true for stimulator cells infected over a large range of multiplicities of gIII$^-$. These findings indicate that a significant proportion of the PrV-specific CTLs produced by DBA/2 mice infected with PrV are directed against gIII.

The finding that in DBA/2 mice gIII is a component of PrV that elicits a significant fraction of the CTL response was corroborated by the results obtained in swine. Thus, in two different species, one of the major proteins of PrV that elicited a CTL response was glycoprotein gIII. Glycoprotein gIII of PrV is a homolog of glycoprotein gC of herpes simplex virus (HSV) type 1 (39), which has been shown to be a dominant viral target antigen in HSV-specific CTL response in mice (7). Our finding that a significant proportion of the CTL response to PrV is directed against glycoprotein gIII is thus consistent with the results obtained with HSV. A shortcoming of our experiments is that the response to gIII of only one pig was ascertained, and it is possible that individual pigs may react somewhat differently. Indeed, recent reports have appeared showing that in different strains of mice, a given viral protein may or may not elicit a major CTL response (13, 48). However, because we have obtained similar results with both DBA/2 mice and swine, it seems likely that gIII is one of the main PrV antigens that induces a cellular immune response.

Although we have concentrated on analyzing the role of nonessential viral glycoproteins in the immune response to PrV and have shown that glycoprotein gIII is important whereas gI, gp63, and gX are not, it is clear that other proteins may be equally important. Thus, analyses of the responses to other viruses have suggested that CTLs may be directed not only against structural but also against nonstructural proteins (23, 36, 52). For example, the immediate-early gene products of human and mouse cytomegaloviruses as well as of HSV type 1 have been shown to constitute major target antigens for antiviral CTL (6, 16, 37). Therefore gIII is probably only one of several viral proteins that elicit a cellular immune response.

It is interesting that a major part of the neutralizing antibodies present in serum obtained from pigs convalescing from PrV infection is directed against glycoprotein gIII (1; F. Zuckermann et al., manuscript in preparation). In view of our finding that gIII appears to be the viral component that elicits a strong CTL response, it appears that gIII may play a central role in the induction of immunity and that therefore it is likely to be important in the induction of a protective immune response. It is clear, therefore, that it should be included as a component of any vaccine used in the control of Aujeszky's disease.

ACKNOWLEDGMENT

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