Pseudorabies Virus Propagated in Rabbit Kidney-Derived RK13 Cells is Neutralized by Natural IgM Antibodies in Normal Swine Serum which Specifically Lyse Host Cells

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ABSTRACT. Pseudorabies virus (PRV) propagated in rabbit kidney-derived RK-13 cells (PRV-RK) was neutralized by serum obtained from specific pathogen-free pigs through the activation of complement. The virus-neutralizing activity of swine serum was lost after treatment with ethylene glycol-bis-aminoethylether-N,N,N',N'-tetraacetic acid (EGTA) or ethylenediaminetetraacetic acid (EDTA). Anti-C1q and anti-IgM antibodies also inhibited virus-neutralizing activity. Though IgG-depleted swine serum neutralized PRV, IgM and IgG-free swine serum lost virus-neutralizing activity. Pre-incubation of swine serum with RK-13 cells, but not with swine kidney-derived CPK cells, at 4°C eliminated the virus-neutralizing activity to PRV-RK. Results indicated that swine serum contained natural IgM against an antigen(s) on the RK-13 cell surface and that this surface antigen was integrated into the PRV envelope during the budding process. Thus the natural IgM in swine serum reacted with the RK-13 antigen on the viral envelope, activated the complement cascade and neutralized the PRV-RK.

KEY WORDS: complement, natural antibodies, PRV.

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We previously reported that pseudorabies virus (PRV) grown in rabbit kidney-derived RK-13 cells was neutralized by swine serum while the same virus grown in porcine kidney-derived CPK cells was resistant [17]. However, the virus-neutralizing activity of swine serum was lost when serum was heat-inactivated at 56°C for 30 min. We speculated that neutralization of PRV was caused by activation of complement via the alternative pathway because of the apparent absence of anti-PRV antibodies in swine serum [17]. We also found that a similar phenomenon was observed when PRV grown in several cell lines was incubated with human serum [13]. PRV grown in several cell lines also showed different resistant levels against rabbit serum [13].

It has been known that human immunodeficiency virus type 1 acquires cell membrane complement regulatory factors on its envelope from cells during the budding process, thereby escaping from complement attack [18]. Therefore it was assumed that RK-13-grown PRV acquired complement regulatory factors from the host rabbit cells and these were less effective against swine complement attack. On the other hand PRV grown in CPK cells acquired swine complement regulatory factors which are more effective regulators against swine complement and thus were resistant to swine complement attack [17].

However, further study revealed that RK-13 cells were lysed by swine serum *via* the activation of complement clas-

sical pathway (Hayashi *et al.*, in preparation). It was found that swine serum contained natural IgM antibodies that specifically reacted with an antigen or antigens on the RK-13 cell surface. Therefore a question arose whether the anti-RK-13 natural IgM in swine serum is responsible for the neutralization of PRV grown in RK-13 cells.

In this study, using swine serum and PRV propagated in RK-13 cells (PRV-RK), we investigated the mechanism for the virus-neutralizing activity of normal serum on viruses grown in other animal-derived cells and demonstrated that swine natural IgM antibodies reacted with an antigen or antigens on the PRV envelope, which was derived from RK-13 cells, and this activated the classical pathway of complement.

MATERIALS AND METHODS

Cells and viruses: Rabbit kidney-derived RK-13 and porcine kidney-derived CPK cells were cultured in Eagle's minimum essential medium (E-MEM, Nissui, Tokyo, Japan) supplemented with 7.5% heat-inactivated fetal calf serum (FCS) and 60 µg/ml kanamycin. Two strains of PRV, PRV (BUK-dl3) and PRV (dlg92dltk), were described earlier [15, 16]. PRV (BUK-dl3) was a thymidine kinase deletion mutant derived from the Bucharest strain of PRV. PRV (dlg92dltk) was derived from PRV (BUK-dl3) with a deletion in the gC gene. These viruses were propagated in CPK cells and kept at –80°C in our laboratory. Before using these viruses for experiments in this study, these viruses were once propagated in RK-13 cells (PRV-RK) and PRVdlgC-RK were obtained by propagating PRV(BUK-

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dl3) or PRV(dlg92dltk) in RK-13 cells, respectively).

Sera: Blood samples were obtained from specific pathogen free (SPF) pigs (age 6 months, Zen-noh Institute of Animal Health, Chiba, Japan) and from healthy Japanese White strain rabbits. Blood samples were allowed to clot at room temperature for 60 min, and pooled sera were aliquoted and stored at -80°C . Neither swine nor rabbit sera used in this study contained antibodies against PRV by ELISA or virus neutralization tests.

Reagents and antibodies: Goat anti-human complement C1q antiserum was purchased from Sigma (Saint Louis, MO). Affinity-purified goat antibody against swine IgM (μ -specific) and peroxidase-labeled goat anti-swine IgM (μ -specific) were purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Peroxidase-labeled rabbit anti-swine IgG (H+L-specific) was purchased from ZYMED Laboratories, Inc. (San Francisco, CA). Polyclonal mouse antisera against RK-13 or CPK cells were prepared as previously described [17].

Plaque-titration of PRV: CPK cells were grown in 24 well plates and incubated at 37°C until monolayers were formed. Medium was removed from the wells, and virus suspension was added at 0.1 ml per well. After 1 hr incubation at 37°C to permit adsorption, the monolayers were overlaid with E-MEM containing 0.5% methylcellulose and 1% FCS. Plaques were allowed to develop for 2 days, fixed with 99.5% ethanol, stained with 0.15% crystal violet, and counted.

Complement virus neutralization assay (CVN assay): A 300 μl reaction mixture contained about 300 PFU of PRV-CPK, PRV-RK, PRVdlgC-CPK or PRVdlgC-RK, swine or rabbit serum as a source of complement, 20 mM HEPES buffer, and E-MEM supplemented with 1% heat-inactivated FCS. The reaction mixtures were incubated at 37°C for 1 hr, and the surviving virus was plaque-titrated using 3 wells of CPK cell monolayers. To examine the inhibitory effect of anti-swine IgM or anti-human C1q antibodies, they were incubated with swine serum at 4°C for 1 hr. These sera were used in the CVN assay as described above.

Calcium and magnesium ions in swine serum were chelated by adding ethylenediaminetetraacetic acid (EDTA) at a final concentration of 10 mM. Calcium ion alone was chelated in 10 mM ethylene glycol-bis-aminoethylether-N,N,N',N'-tetraacetic acid (EGTA) containing 2 mM MgCl_2 . With the CVN assays using these chelated sera, 3×10^4 PFU of PRV-RK or PRVdlgC-RK were incubated with these sera at 37°C for 1 hr, and the reaction mixture was diluted 1:100 with E-MEM and then plaque-titrated using 3 wells of CPK cell monolayers.

Pre-absorption of swine serum with cells: A 500 μl reaction mixture containing 20% swine serum and 2.5×10^7 RK-13 or CPK cells in phosphate buffered saline (PBS) was incubated on ice for 1 hr and centrifuged, and the cell-free supernatant serum was used in the CVN assay.

Preparation of IgG-depleted swine serum: One ml of swine serum was applied to a pre-packed protein A column (HiTrapTM protein A HP, Amersham Biosciences, Upp-

sala, Sweden) and eluted with PBS. The pass-through fractions were pooled, aliquoted, and stored at -80°C .

Preparation of rabbit anti-swine IgM antibodies: Fifteen ml of saturated ammonium sulfate was added drop-wise to 30 ml swine serum, and this was kept at 4°C for 2 hr and centrifuged at $10,000 \times g$ for 30 min. The supernatant was discarded, and precipitates were dissolved in 10 ml PBS and centrifuged at $10,000 \times g$ for 30 min. The supernatant was applied to a Bio-Gel A-0.5m gel (Bio-Rad Laboratories, Hercules, CA) column (2.5×90 cm) and eluted with PBS. The fractions containing IgM were located by ELISA test using peroxidase-labeled goat anti-swine IgM (μ -specific) (data not shown). These fractions were then pooled, concentrated by ultra-filtration by a centrifugal filter device (ULTRAFREE-15, MILLIPORE, Bedford, MA), and re-chromatographed by the same Bio-Gel A-0.5m column. The IgM fractions were pooled and used for immunizing rabbits.

New Zealand white rabbits were immunized with 1.5 mg of swine IgM with complete Freund's adjuvant per rabbit and 3 weeks later with Freund's incomplete adjuvant. Two more boosters were given at two weeks intervals with the same amount of swine IgM with Freund's incomplete adjuvant. One week after the final immunization, blood samples were collected from the rabbits, and rabbit anti-swine IgM sera were prepared.

Preparation of protein A bead antibody affinity column: Rabbit anti-swine IgM antibody was immobilized on protein A beads according to Gersten and Marchalonis [11]. Briefly, 2 ml of rabbit anti-swine IgM serum was mixed with 2 ml suspension of protein A-Sepharose 4B beads (SIGMA, St. Louis, MO), and this was incubated at room temperature for 1 hr with gentle rocking. The beads were washed twice with 10 ml of 0.2 M sodium borate buffer (pH 9.0) and re-suspended in 10 ml of the same buffer. To the suspension, solid di-methylpimelimidate was added to bring the final concentration to 20 mM, and this was mixed for 30 min at room temperature. The reaction was stopped by washing the beads once in 0.2 M ethanolamine (pH 8.0), and this was then incubated for 2 hr at room temperature in 0.2 M ethanolamine.

Preparation of IgM and IgG-depleted swine serum: Protein A beads coupled with rabbit anti-swine IgM antibodies as described above were poured into a small column to a bed volume of 1.5 ml and washed extensively with PBS. To the top of the column, 0.5 ml of swine serum was applied, and this was eluted with PBS. The pass-through protein fraction was collected, aliquoted, stored at -80°C , and thawed immediately before use as a source of IgM and IgG-depleted swine serum. The fraction was checked for the absence of anti-swine IgG or IgM antibodies by ELISA test (data not shown).

RESULTS

Characterization of the virus-neutralizing mechanism of swine serum: Previously we demonstrated that PRV propa-

Fig. 1. Effect of EGTA and EDTA on the virus-neutralizing effect of swine serum on PRV-RK (A) or PRVdlgC-RK (B). To inactivate both classical and alternative complement pathways, EDTA was added to swine serum at a final concentration of 10 mM. To inactivate only the classical complement pathway, 10 mM EGTA and 2 mM $MgCl_2$ were added. Viruses were incubated with 0 to 10% swine serum pre-treated with EDTA or EGTA at 37°C for 1 hr and then plaque titrated. The survival rate was calculated as the percentage of the number of plaques of the control (the same virus incubated without serum). Vertical lines represent S.D.

gated in RK-13 cells was neutralized by normal swine serum. Since this virus-neutralizing activity was eliminated by heating the serum at 56°C for 30 min, it was assumed that the activation of complement was responsible for the virus-neutralizing activity [17]. To determine whether the virus-neutralizing effect of swine serum was activated via the complement classical pathway or the alternative pathway, PRV-RK and PRVdlgC-RK were reacted with swine sera pretreated with EDTA or EGTA. It has been known that both Mg^{2+} and Ca^{2+} ions are required to activate the classical pathway of complement, while Mg^{2+} but not Ca^{2+} is required to activate the alternative pathway. Therefore EDTA blocks both pathways, and EGTA (in the presence of Mg^{2+}) blocks only the classical pathway [5]. As shown in Fig. 1, 70% of PRV-RK and 80% of PRVdlgC-RK were neutralized by 10% normal swine serum as compared with control (0%

Fig. 2. Effect of anti-human C1q antibody on the sensitivity of PRV-RK to swine serum. Swine serum was incubated with 0 to 6 mg/ml of goat anti-human C1q antibody at 4°C for 30 min., and viruses were then added to the mixture. The final concentration of the serum is 10%. Survival rate was calculated as described in Fig. 1. To check the toxicity of anti-C1q antibody, viruses were mixed with 6 mg/ml of anti-C1q antibody in the absence of serum.

serum concentration). On the other hand, EDTA or EGTA treatment completely eliminated the virus-neutralizing effect of swine serum against PRV-RK (Fig. 1A) and PRVdlgC-RK (Fig. 1B). Therefore it can be speculated that the virus-neutralizing activity of swine serum was due to the activation of the classical pathway of complement.

To further confirm that the virus-neutralizing activity of swine serum was due to the activation of the classical pathway of complement, the effect of anti-C1q antibody was tested in the CVN assay. Complement component C1 is a large, multimeric protein complex composed of C1q, C1r, and C1s subunits. C1 binds to two or more Fc portions of antibody that has bound antigen and initiates the complement classical pathway [19]. Figure 2 shows the effect of anti-human C1q antibody on the virus-neutralizing activity of swine serum against PRV-RK (Fig. 2) in the presence of 10% swine serum. Addition of anti-C1q antibodies decreased the virus-neutralizing activity of swine serum in a dose-dependent manner. Although the anti-C1q antibody used in this study was anti-human C1q, it was confirmed that this antibody cross-reacted with swine C1q molecule by Western blot analysis (data not shown).

IgM antibodies as the activator of the classical pathway: Since the classical pathway of complement activation is triggered by either IgG or IgM antibodies, swine serum must contain IgG or IgM which specifically reacts with PRV-RK or PRVdlgC-RK. Previous study revealed that normal swine serum contains natural IgM antibodies that react specifically with RK-13 cells and activate the classical pathway of complement (Hayashi *et al.*, in preparation). It may be possible that the virus-neutralizing activity of swine serum against PRV-RK or PRVdlgC-RK uses the same natural IgM antibodies against RK-13 cells. This assumption was confirmed by the observation that after incubating swine serum with anti-swine IgM (μ) antibody at 4°C, swine

Fig. 3. Effect of anti-swine IgM antibody on the sensitivity of PRV-RK (A) or PRVdlgC-RK (B) to swine serum. Swine serum was incubated with 0 to 0.3 mg/ml of anti-swine IgM antibody at 4°C for 30 min, and viruses were then added to the mixture. The final concentration of the serum is 10%. Survival rate was calculated as described in Fig. 1. To check the toxicity of anti-IgM antibody, viruses were mixed with 0.3 mg/ml of anti-IgM antibody in the absence of serum.

serum lost its virus-neutralizing activity against PRV-RK and PRVdlgC-RK. As shown in Fig. 3, anti-swine IgM antibody protects PRV-RK (Fig. 3a) and PRVdlgC-RK (Fig. 3b) from neutralization by swine serum. Since the anti-swine IgM (μ) antibody used in this study did not react with swine IgG, contribution of IgG to the virus-neutralizing effect of swine serum seemed to be negligible. Indeed IgG-depleted swine serum still retained the virus-neutralizing effect (Fig. 4).

IgM and IgG antibodies were depleted from swine serum by using an affinity column as described in Materials and Methods. As shown in Fig. 5, IgM, IgG-depleted swine serum completely lost its virus-neutralizing activity against PRV-RK and PRVdlgC-RK. To check whether this IgM, IgG-depleted serum still retained complement components for the classical pathway, CPK cells sensitized by mouse anti-CPK antibody were incubated with this serum (data not shown). It was found that the IgM, IgG-depleted swine serum contained complement components for the classical

Fig. 4. Virus-neutralizing effect of IgG-depleted swine serum on PRV-RK (A) or PRVdlgC-RK (B). Viruses were incubated with 0 to 20% IgG-depleted swine serum at 37°C for 1 hr. Survival rate was calculated as described in Fig. 1.

pathway (data not shown).

The effect of pre-absorption of swine serum with RK-13 or CPK cells: When RK-13 cells were incubated with swine serum at 4°C, no cytotoxicity was observed. At this temperature IgM was absorbed onto the cell surface, but complement was not activated (data not shown). Swine serum was incubated with RK-13 or CPK cells at 4°C in order to remove IgM that has affinity to RK-13 or CPK cells. The cell-free supernatant was tested for virus-neutralizing activity against PRV-RK or PRVdlgC-RK at 37°C. As shown in Fig. 6, swine serum pre-treated with RK-13 cells lost its virus-neutralizing activity to PRV-RK. On the other hand, swine serum pre-treated with CPK did not lose its neutralizing activity to PRV-RK (Fig. 6a). The same results were observed when PRVdlgC-RK were incubated with each serum (Fig. 6b).

DISCUSSION

Electron microscopic analysis of alphaherpesviruses revealed that after intranuclear assembly, nucleocapsids bud at the inner leaflet of the nuclear membrane, resulting in enveloped particles in the perinuclear space. Egress from the perinuclear cisterna occurs by fusion of the primary envelope with the outer leaflet of the nuclear membrane. The resulting intracytoplasmic naked nucleocapsids acquire

Fig. 5. Virus-neutralizing effect of IgM, IgG-depleted swine serum on PRV-RK (A) or PRVdlgC-RK (B). Viruses were incubated with 0 to 10% IgM, IgG-depleted swine serum at 37°C for 1 hr. Survival rate was calculated as described in Fig. 1.

their final envelope downstream in the secretory pathway [2, 4, 10, 12]. Indeed, our previous study revealed that purified PRV-RK virion contains not only its envelope glycoprotein gC but also RK-13 cellular proteins [17]. Moreover, it was suggested that RK-13 cells were lysed by normal swine IgM antibodies via classical complement pathway (Hayashi *et al.*, in preparation). Therefore, it is probable that the herpesvirus envelope contains some components of the cellular membrane. In this study, we found that natural IgM in swine serum reacted with PRV-RK, activated complement via the classical pathway, and neutralized virus. The fact that swine serum loses its virus-neutralizing activity by pre-exposing serum to RK-13 cells suggests that the same antigen(s) on the surface of RK-13 cells exists on the envelope of PRV-RK. This antigen(s) on the viral envelope is the target of the natural IgM antibodies in swine serum. Since PRV grown in CPK cells (PRV-CPK, PRVdlgC-CPK) did not contain the RK-13 cell surface antigen, the natural IgM in swine serum did not react with PRV-CPK particles, and thus PRV-CPK was not neutralized by swine serum (data not shown). PRV-CPK may contain CPK cell-derived complement regulatory factors in the viral envelope, but these probably play only a minor role in resisting the neutralizing effect of swine serum.

It is known that the α -gal epitope is a common carbohydrate structure identified on the surface of cells of almost all mammals except Old World primates and the anti- α -gal epitope natural antibodies exist in the normal serum of Old World primates including humans [19]. We prepared PRV

Fig. 6. The effect of pre-absorbing swine serum with RK13 or CPK cells on PRV-RK (A) or PRVdlgC-RK (B). Swine serum was incubated with RK13 or CPK cells at 4°C for 1 hr, and 0 to 5% of the cell-free supernatant serum was tested for virus-neutralizing activity against PRV-RK or PRVdlgC-RK at 37°C. Survival rate was calculated as described in Fig. 1.

grown in cells with no- or low-level expression of α -gal epitope such as Vero cells (PRV-Vero). Though PRV-CPK was neutralized by normal human serum, PRV-Vero was not neutralized. ELISA was carried out using purified PRV-CPK and PRV-Vero virions, α -gal epitopes were detected on PRV-CPK virion, and neither on PRV-Vero [13]. In the same way, it is suggested that PRV-RK virion also contains the natural IgM epitope on its envelope. Although the reaction of anti- α -gal epitope natural antibodies to PRV-CPK was directly proved by ELISA [13], we failed to detect the natural IgM in swine serum reacting to PRV-RK by ELISA, Western blotting analysis and immuno-precipitation (data not shown). So far we don't know the reason of the failure. The effect may not be due to antibodies reacting with only one epitope as like the anti α -gal antibodies but due to sum of many different antibodies, of which epitopes also varied. The amount of each natural IgM in swine serum reacting PRV-RK might be much less than that of anti- α -gal epitope natural antibody.

It would appear that the lysis of RK-13 cells and the neutralization of PRV-RK by swine serum are triggered by the same antigen(s) and natural IgM antibodies, but the mechanisms for cell lysis and virus neutralization by complement may be different. It has commonly been known that cell lysis by complement is due to the formation of a membrane attack complex (MAC). Cell-associated C5 convertase cleaves C5 and generates C5b, which becomes bound to the convertase. C6 and C7 bind sequentially, and the C5b, 6, 7 complex becomes directly inserted into the lipid bilayer of the plasma membrane, followed by stable insertion of C8. Up to 15 C9 molecules may then polymerize around the complex to form pores in the membrane [1]. On the other hand, Friedman et al. demonstrate that complement component C8 and terminal lytic activity are not required to neutralize herpes simplex virus type 1 (HSV-1) [8]. Our preliminary experiments also indicated that virus neutralization by natural IgM did not require the formation of MAC (data not shown). It may be possible that deposition of C3 on virus particles, triggered by natural IgM, is enough to abolish the infectivity of PRV.

It has been known that glycoprotein C (gC) of alphaherpesviruses, such as HSV-1, serves as an inhibitor of activation of the complement cascade by binding complement component C3b and by blocking the binding of properdin and C5 to C3b [3, 6, 7, 14]. Although statistical differences were not detected, the gC-deletion mutant PRVΔgC-RK was always more sensitive to 10% swine serum treatment (Figs. 1, 2, 3 and 5). It may be due to the effect of the deletion of gC.

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