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Horses infected with equine infectious anemia virus (EIAV) have recurrent episodes of viremia which are eventually controlled, but the immune mechanisms have not been identified. Antibodies were detected to the surface of EIAV-infected cells within 1 month postinfection and remained for at least 3.5 years postinfection. These antibodies recognized cell surface-exposed envelope (Env) glycoproteins, but could not mediate antibody dependent cellular cytotoxicity (ADCC) using EIAV-WSU5-infected equine kidney (EK) cells as targets and peripheral blood mononuclear cells (PBMC) or polymorphonuclear cells (PMN) as effector cells. Furthermore, purified IgG antibodies from horses infected with either EIAV-WSU5 or EIAV-Wyo did not mediate ADCC of infected target cells. Armed effector cells could not be detected in infected horse blood nor could effector cells be prearmed by incubation with serum antibodies to cell surface antigens. The use of EIAV-WSU5-infected equine macrophages as target cells did not result in ADCC. In contrast, serum antibody from EHV-1 vaccinated horses and PBMC or PMN as effector cells caused ADCC of EHV-1-infected EK cells. These results indicate that ADCC is not involved in the control of EIAV in carrier horses.

INTRODUCTION

EIAV is a lentivirus of horses that causes recurrent episodes of cell-free plasma viremia with concurrent fever, thrombocytopenia, and anemia. Clinical episodes occur over the first year of infection with each episode being associated with an antigenically distinct virus isolate as defined by virus neutralizing antibody assays (Kono *et al.,* 1973) and the control of the initial viremic episode is mediated by lymphocyte responses as indicated by the inability of combined immunodeficient Arabian foals to terminate viremia (Perryman *et al.,* 1988). Most horses control EIAV and develop a carrier state that also appears to be associated with immunological control as indicated by the recrudescence of viremia and disease in carrier horses treated with cyclophosphamide (Kono *et al.,* 1976) or dexamethasone (Kono *et al.,* 1976; Tumas *et al.,* 1994b). Following infection of horses with EIAV, neutralizing antibodies (Kono, 1968; O'Rourke *et al.,* 1989), complement-fixing antibodies (Kono *et al.,* 1966), complement-inhibiting antibodies (McGuire *et al.,* 1971), and cytotoxic T lymphocytes (McGuire *et al.,* 1994a) have been identified. The immune mechanism responsible for the control of viremia has not been identified, but passive transfer of heat-inactivated plasma from carrier horses given prior to infection resulted in decreased clinical illness and an increase in the number of days postinfection when seroconversion occurred (Rushlow *et al.,* 1990).

The presence of unique cell surface antigens following infection of equine dermal (ED) cells with EIAV (McGuire and Crawford, 1978) and serum antibodies to the EIAV Env glycoproteins (O'Rourke *et al.,* 1988; Perryman *et al.,* 1990) indicated that antibody dependent cellular cytotoxicity (ADCC) may play a role in EIAV control. However, two papers have been published on ADCC in horses infected with EIAV with conflicting results. A lack of ADCC activity was originally reported (Fujimiya *et al.,* 1979) followed by a second report that detected ADCC activity only during the first viremic episode following infection (Gerencer *et al.,* 1989). Studies from other lentiviral infections have determined that ADCC mediating antibodies are present in HIV-infected humans (Kenealy *et al.,* 1987; Koup *et al.,* 1989; Tanneau *et al.,* 1990; Nixon *et al.,* 1992), HIV-infected chimpanzees (Belo *et al.,* 1991), SIV-infected macaques (Ohkawa *et al.,* 1995), and sheep infected with maedi-visna (Tuboly *et al.,* 1991). ADCC mediating antibodies predominantly recognize the viral Env glycoproteins, surface unit (SU), and transmembrane (TM) (Kenealy *et al.,* 1987; Koup *et al.,* 1989; Tanneau *et al.,* 1990) in HIV-infected humans, and the epitopes recognized are in the conserved regions of the SU and TM (Nixon *et al.,* 1992).

The identification of immune mechanisms which can function in the face of continually changing viral protein epitopes will increase the understanding of lentivirus/host interactions and may lead to the identification of immune responses capable of prolonging the asymptomatic stage. The purpose of this research was to identify

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the viral proteins expressed on the surface of EIAV-infected equine kidney (EK) cells and to analyze different IgG isotype antibodies binding to these cell surface-exposed viral proteins for their ability to mediate ADCC activity.

MATERIALS AND METHODS

Horses

Effector cells were isolated from blood from Arabian mares between the ages of 18 and 24 years maintained at Washington State University. Horses infected with EIAV-WSU5 were mixed breed ponies (McGuire *et al.,* 1994a). Horses infected with EIAV-Wyo were infected by iv injection of whole blood from EIAV-Wyo-infected horses. All horses were maintained according to institutional animal care and use committee guidelines.

Cell cultures

All EK cells used in these experiments were from a kidney biopsy of an adult mixed breed pony or a kidney from a normal Arabian foal taken at necropsy. EK cells were maintained as described previously (O'Rourke *et al.,* 1988) in minimal essential medium (MEM) supplemented with 10 m*M* HEPES, 1.75 g/L sodium bicarbonate, and 5% calf serum.

EK cells were infected with 1 m.o.i. of EIAV-WSU5 in a small amount of medium and incubated at 37° overnight. Virus was removed the next day and fresh medium added. Cells were not used for any experiments until $\geqslant$6 days postinfection because live-cell flow cytometry experiments indicated this was the first day of maximal surface antigen expression of EIAV proteins. EK cells were infected with 0.17 m.o.i. of EHV-1. Viruses were added to 150-cm^2 flasks in 10 ml of medium and cells were used 20 hr later.

Macrophage cultures were started from heparinized blood collected from normal, uninfected adult horses. Peripheral blood mononuclear cells (PBMCs) were obtained using Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) as described (Wyatt *et al.,* 1988) and macrophages were isolated (Freundlich and Avdalovic, 1983; Maury, 1994). Macrophages were maintained as described (Maury, 1994) and infected with 1 m.o.i. of EIAV-WSU5, as done with EK cells. The macrophage cultures were characterized by morphology and the ability of $>$93% of the cells to phagocytize latex beads (Tumas *et al.,* 1994a). Live-cell flow cytometry determined that maximal surface antigen expression occurred at 4 days postinfection and subsequent experiments using macrophages were done at this time.

Fluorescent flow cytometry

Normal EK cells, EIAV-infected EK cells, and EHV-1-infected EK cells were lifted from 150-cm^2 flasks with 4 ml ATV, placed in 50-ml conical centrifuge tubes with 15 ml of growth medium, and incubated in a shaking incubator (37°, 200 rpm) for 2 hr. This maximized the reexpression of cell surface antigens following trypsinization, while minimizing cell death. Cells were labeled for flow cytometry (Tumas *et al.,* 1994a) with serum, and bound antibody was detected with goat anti-equine immunoglobulin antibodies conjugated to 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (FITC) (Chemicon International, Inc., Temecula, CA). All data using EK cells were acquired using unfixed cells in the presence of 1 μg/ml propidium iodide (Sigma Chemical Co.). Macrophages were scraped from 25-cm^2 tissue culture flasks and incubated with FITC-conjugated serum immunoglobulin from either a normal horse or an EIAV-Wyo infected horse. Live macrophages were identified by fluorescence following exposure to dihydroethidium (Molecular Probes, Inc., Eugene, OR). Therefore in all flow cytometry experiments, only live cells were analyzed on a FACSort or FACScan equipped with a Power Macintosh 7100/80 computer and CellQuest software (Becton-Dickinson, San Jose, CA).

Target cells for ADCC assays

EK cells were labeled overnight with ^{51}Cr in tissue culture flasks and used as targets. Labeling medium was growth medium containing 50 μCi/ml ^{51}Cr. Normal and EHV-1-infected EK cells at the same passage as the EIAV-infected EK cells were used as negative and positive controls, respectively. Target EK cells were washed once in the flask with MEM without serum, lifted with ATV, and washed three times with MEM plus 5% BCS in 50-ml conical centrifuge tubes. Labeled EK cells were resuspended in assay medium (RPMI 1640 containing 20 m*M* HEPES, 1.75 g/L sodium bicarbonate, and 10% FCS) at 2.66 $\times$ 10^5 cells/ml and 75 μl was added to each assay well.

ADCC assays

PBMC and polymorphonuclear cells (PMN) isolated from normal, uninfected adult horses were used as effector cells in all ADCC assays. PBMC and PMN were isolated from whole blood and red blood cells were lysed using 0.87% Tris–ammonium chloride, pH 7.2 (Wyatt *et al.,* 1988). Effector cells were resuspended in assay medium and the viability was determined using trypan blue dye exclusion. Replicates of six with effector to target cell ratios of 50:1 or 100:1 were incubated for 8 or 17 hr, and 50% of the supernatant was used to determine ^{51}Cr release in a β-plate reader (Wallac, Gaithersburg, MD). The formula used to determine SE of the percentage of specific lysis took into account the variability occurring in both the test and the control wells (Siliciano *et al.,* 1985). ADCC assays using macrophages as target cells were done in a similar manner except that experimental and control wells were done in replicates of three. Spontaneous release defined as the mean of wells containing

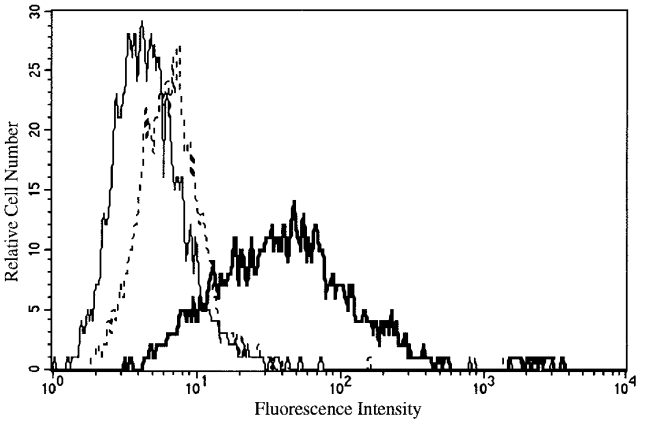

FIG. 1. Live-cell flow cytometry of normal and EIAV-infected EK cells incubated with pre- and post-EIAV-WSU5 infection horse serum samples. The overlaid histogram shows surface staining of infected EK cells incubated in the presence of serum collected prior to infection (---), uninfected EK cells incubated with serum collected 4 months postinfection (—), and infected EK cells incubated with serum collected 4 months postinfection (▬). Infected cells were used at >6 days postinfection and dead cells were gated out using propidium iodide as the indicator.

target and effector cells in the absence of serum or equine IgG was reduced by using macrophage culture medium (DMEM supplemented with 1.75 g/L sodium bicarbonate, 20 m*M* HEPES, 15% horse serum, and 15% fetal calf serum).

RESULTS

Antibody responses to EIAV Env glycoproteins on the surface of EK cells

Live-cell flow cytometry was used to determine if infected EK cells expressed EIAV-WSU5 antigens on their surface as reported for ED cells infected with the prototype strain of EIAV (McGuire and Crawford, 1978). EIAV-infected EK cells had surface antigen expression as demonstrated by a significant increase in mean peak channel fluorescence over uninfected EK cells (Fig. 1) when reacted with serum collected 1 year post-EIAV-WSU5 infection, but not when reacted with preinfection serum. Uninfected EK cells did not react with either serum. Live-cell flow cytometry using EK cells infected with recombinant vaccinia virus expressing the EIAV Env proteins (McGuire *et al.,* 1994a), with EIAV Gag proteins plus the protease, or with no EIAV proteins (McGuire *et al.,* 1994b) determined that only EIAV Env proteins were expressed on the surface of EK cells. The serum used had antibodies to the EIAV Gag proteins detected by agar gel immunodiffusion.

Sequential serum samples from EIAV-WSU5-infected horses were titered using live-cell flow cytometry to determine if antibodies to surface epitopes of the Env glycoproteins were present from 1 to 24 months after infection. The antibody titer was the reciprocal of the serum dilution necessary for 50% reduction in signals using EIAV-WSU5 infected EK cells (Fig. 2). Titers were maximal within 1 month postinfection and remained at near maximal levels over the 2-year observation period. Serum samples from a third horse, H507, had titers ranging from 2100 to 2500 between 1.5 and 3.5 years postinfection. Following detection of serum antibodies binding cell surface EIAV Env glycoproteins, serum samples were tested for ADCC activity.

ADCC activity of EIAV serum antibodies

An ADCC assay was developed and several variables were tested including serum dilution, incubation time, target to effector cell ratios, and the effect of preincubation of serum antibodies with effector or target cells. At a serum dilution of 1:30, no ADCC activity was detected in any of the seven post-EIAV-WSU5 infection serum samples from H507, H521, or H525. Purified PBMC and PMN were used as effector cells because the neutrophil is the primary effector cell for ADCC in the horse (Fujimiya *et al.,* 1979; Stokes and Wardley, 1988) while peripheral blood monocytes and an unidentified population of lymphocytes can also mediate ADCC (Stokes and Wardley, 1988). Altering the incubation period of the assay, the effector to target cell ratio, and the serum dilution did not result in the detection of ADCC activity. Preincubation of serum with target or effector cells did not result in specific lysis of targets nor were prearmed effector cells detected. To verify that the effector cells could mediate ADCC of EK cell targets, a control assay was developed using an equine herpes virus, EHV-1 (Stokes and Wardley, 1988).

EHV-1 control ADCC assay

Live-cell flow cytometry was used to demonstrate the presence of unique cell surface antigens following infection of EK cells by EHV-1 and serum antibodies to these

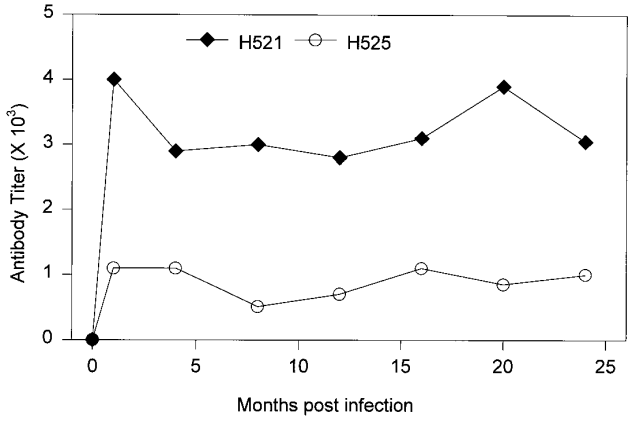

FIG. 2. Titration of horse serum antibodies to EIAV-WSU5 cell surface antigens using live-cell flow cytometry. Normal and infected EK cells were incubated in the presence of serial dilutions of serum from EIAV-WSU5-infected horses at 0, 1, 4, 8, 12, 16, 20, and 24 months postinfection. Serum dilutions resulting in binding to cell surface antigens on 50% of the infected cells were calculated and the inverse of this serum dilution was the titer.

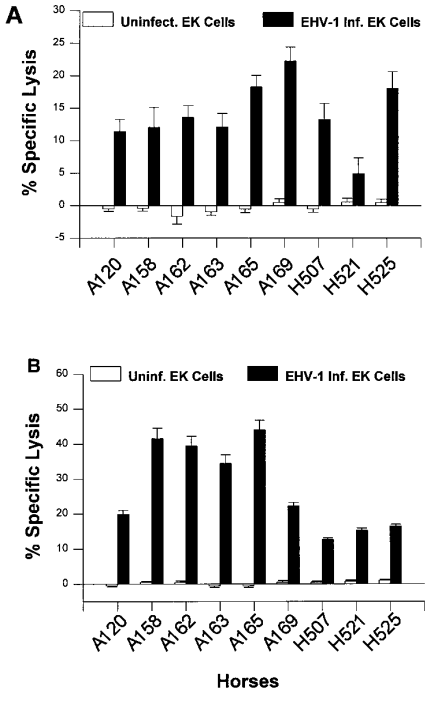

FIG. 3. ADCC of normal and EHV-1-infected EK cells by serum samples from horses vaccinated with an EHV-1 vaccine. Assays were done using as effector cells either PBMC (A) or PMN (B) from a normal adult horse. The effector to target cell ratio was 100:1, incubation time of the assay was 8 hr, and spontaneous release was below 20% of total release.

antigens following EHV-1 vaccination of horses. Serum from six normal horses and three EIAV-infected horses, all vaccinated against EHV-1, had antibodies to the surface of EHV-1-infected cells. These sera were tested at a 1:30 dilution against EHV-1-infected EK cells (Stokes and Wardley, 1988) and all mediated EHV-1-specific ADCC activity (Fig. 3), although the specific lysis varied among horses. The absence of ADCC against EIAV-infected EK cells combined with the ability of the same serum samples and effector cells to mediate the lysis of EHV-1-infected EK cells indicated that neither the assay format nor the effector cells were responsible for the lack of killing.

ADCC using highly purified equine IgG

Horse IgG(T) antibodies, a subtype of equine IgG, inhibit complement activation (McGuire *et al.,* 1971) and ADCC activity at even low concentrations (Fujimiya *et al.,* 1979). The presence of Env protein-specific IgG(T) antibodies in the serum samples of EIAV-infected horses (O'Rourke *et al.,* 1988) could inhibit ADCC activity. IgG was purified from serum samples from two EIAV-infected horses (McGuire *et al.,* 1979) and an ELISA was used to determine the purity of the IgG fractions. Monoclonal antibody 2/18.2.1 was used as a positive control because it bound only to equine IgG at all concentrations tested. Contaminating IgG(T) was detected using goat anti-equine IgG(T) polyclonal serum (Bethyl Laboratories, Inc., Montgomery, TX). A standard curve made with purified IgG spiked with serial dilutions of IgG(T) was used to quantitate the amount of IgG(T) in fractions from the DEAE column. All IgG fractions had less than 0.05% contaminating IgG(T) and still reacted with the Env proteins expressed on the surface of EIAV-WSU5-infected EK cells by live cell flow cytometry.

Purified IgG fractions from EIAV-WSU5-infected horses were tested for ADCC activity and no killing was detected using dilutions of 1:30, 1:300, and 1:3000 of 20 mg/ml IgG. Serum and purified IgG antibodies from three horses infected with the EIAV-Wyo strain were examined to determine if a more virulent virus isolate would induce IgG antibodies mediating ADCC activity. Despite reactivity to EIAV Env glycoproteins expressed on the surface of infected cells, no killing was detected in these assays using whole serum or purified IgG.

ADCC assays using EIAV-infected equine peripheral blood-derived macrophages

To ensure that the lack of ADCC activity in previous assays was not due to use of the wrong target cell, blood-derived macrophage cultures were used as target cells. While EK cells support the growth and propagation of EIAV-WSU5, the primary cell infected *in vivo* is the tissue macrophage (Sellon *et al.,* 1992). Peripheral blood mononuclear cells were purified and infected with EIAV. Macrophages had detectable EIAV cell surface antigen expression by live-cell flow cytometry using polyclonal horse serum antibodies directly conjugated to FITC (Fig. 4). A twofold increase in peak channel fluorescence was apparent when EIAV-infected macrophages were incubated in the presence of post-EIAV infection serum. Even

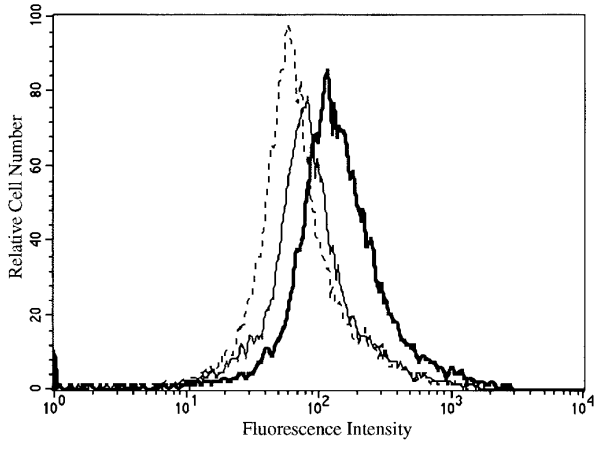

FIG. 4. Live-cell flow cytometry to demonstrate the presence of EIAV antigens on the surface of macrophages derived from the peripheral blood. EIAV antigens were detected using IgG from a horse infected with EIAV-Wyo conjugated to FITC. As a control, IgG from an uninfected horse was conjugated to FITC using the same protocol. Normal macrophages incubated with FITC-conjugated IgG from an EIAV-Wyo-infected horse (---); EIAV-WSU5-infected macrophages incubated with FITC conjugated IgG from an uninfected (—) or EIAV-Wyo infected horse (▬).

though the fluorescence was less than that on infected EK cells (Fig. 1), the increase on infected macrophages was reproducible and statistically significant ($P < 0.001$) by the Kolomogorov–Smirnov test (Young, 1977) when compared to the same serum immunoglobulins incubated with uninfected macrophages or FITC-conjugated normal horse serum immunoglobulins incubated with uninfected or EIAV-infected macrophages.

Infected macrophage targets were labeled with ^{51}Cr overnight and incubated with serum and freshly isolated PMN were added. Pre-EIAV infection serum and serum from three horses infected with EIAV-WSU5 for 1 year were tested for ADCC activity. When no ADCC activity was detected using whole serum, purified IgG from one horse was tested for ADCC activity and again, no specific killing was detected.

DISCUSSION

ADCC has been described following several lentiviral infections with no clear understanding of its role in controlling viral burden or immunopathogenic processes. This work was done to define ADCC mediating antibodies following EIAV infection so that their role could be determined by passive antibody transfer experiments. Previous work characterizing ADCC activity in horses determined that ADCC mediating antibodies could be induced following immunization of horses with sheep red blood cells (Fujimiya *et al.,* 1979) or EHV-1 (Stokes and Wardley, 1988). Further, it was demonstrated that affinity-purified equine IgG antibodies mediate ADCC whereas this activity can be blocked by the presence <13% affinity-purified equine IgG(T) (Fujimiya *et al.,* 1979). However, ADCC activity against EIAV-infected ED cells could not be demonstrated. Later, ADCC mediating antibodies were demonstrated in EIAV-infected horses during the initial viremic episode with specific lysis increasing until 8 days postinfection (Gerencer *et al.,* 1989). This work used peripheral blood lymphocytes isolated from the same horses stimulated with phytohemaglutinin without the addition of virus as the target cell population. Because the primary infected cell *in vivo* is the tissue macrophage (Sellon *et al.,* 1992) and peripheral blood monocytes express only low levels of viral proteins (Maury, 1994), it is unclear what this assay measured.

It was our assumption that ADCC antibodies could be demonstrated in EIAV-infected horse serum by using a more susceptible target cell. EK cells (Montelaro *et al.,* 1982) are used for virus production and cytotoxicity assays because of the increased number of susceptible/permissive cells. In this paper, 72% of the EK cells were infected with EIAV-WSU5 using live-cell flow cytometry compared with ED cultures where 30% of the cells are infected using fluorescent antibody on fixed cells (Klevjer-Anderson *et al.,* 1979). The increase in the number of EK cells expressing EIAV surface antigen recognized by antibodies and work previously demonstrating ADCC activity against EHV-1-infected EK cells (Stokes and Wardley, 1988) made these cells a likely target for lysis by ADCC.

Serum samples were collected from three EIAV infected horses at various times postinfection. Flow cytometry results demonstrated that horses produced antibody to EIAV-infected cell surface epitopes that were detectable for at least the first 3.5 years of infection. ADCC assays using sequential serum samples from these horses resulted in no specific lysis of EIAV-infected EK target cells using either PBMC or PMN as effector cells regardless of effector to target cell ratios, length of incubation time, and serum dilution. Furthermore, the use of highly purified IgG from horses infected with EIAV-WSU5 or EIAV-Wyo strains resulted in IgG antibody binding to target cells, but no specific lysis using either PBMC or PMN as effector cells. A control ADCC assay was developed using the same EK target cells, the same effector cells, and serum samples from the same EIAV-infected horses vaccinated against EHV-1. Specific killing of EHV-1-infected target EK cells occurred with no significant killing of uninfected target cells by incubation with both PBMC and PMN. These results confirmed that the target cells were not resistant to ADCC and that the effector cells were functional.

The use of peripheral blood-derived macrophages as target cells in these ADCC assays resulted in target cells more closely mimicking the *in vivo* situation where the macrophage is the infected cell (Sellon *et al.,* 1992). Initial studies indicated that EIAV-infected macrophages expressed viral antigens on their surface at 2 days postinfection and EIAV antigen density on the surface of infected cells increased, reaching maximal levels at 4 days postinfection. Incubation of EIAV-infected equine macrophages with IgG antibodies in the presence of PBMC or PMN did not result in detectable ADCC activity.

While we have demonstrated EIAV antigens on the surface of infected EK cells and peripheral blood-derived macrophages, have reacted these cells with IgG antibody to the exposed EIAV antigens, and have used effector cells (either PBMC or PMN) shown to work in another ADCC system, no ADCC activity was detected. The horses were followed over the course of the infection, from acute disease to the carrier state, without detecting ADCC mediating antibodies. Although we do not know why IgG antibodies cannot mediate ADCC, it may be due to the number and the spacing of EIAV Env protein molecules on the target cell or to the antibody isotype within the IgG fraction. Whatever the explanation, these results indicate that ADCC does not play a role in the control of lentiviral replication seen in EIAV following viremic episodes or during the carrier state.

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