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Short communication

Cell-mediated immunity to pseudorabies virus: cytolytic effector cells with characteristics of lymphokine-activated killer cells lyse virus-infected and glycoprotein gB- and gC-transfected L14 cells

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We examined cytolytic cells that lyse pseudorabies virus (PRV)-infected cells in pigs. *In vitro* stimulation of peripheral blood mononuclear cells from PRV-immune pigs with live PRV generated cells that lysed PRV-infected immortalized B cells. Several lines of evidence indicated a major contribution of non-major histocompatibility complex (MHC)-restricted cytolytic cells, which displayed characteristics of natural killer (NK) or lymphokine-activated killer cells: cytolysis was non-MHC-restricted, depended on $CD2^+CD4^-CD8^{bright-}$ (or $CD2^+CD4^-CD8^{dull+}$) cells, was strongly augmented by *in vitro* antigenic stimulation and was not limited to virus-infected cells, i.e. the NK cell-susceptible target cell line K562 was also lysed. Cytolytic cells were also generated by *in vitro* antigenic stimulation with UV-inactivated PRV. Target cells transfected with and stably expressing PRV gB or gC were lysed to the same degree as PRV-infected target cells.

We examined the immune response of pigs to the alphaherpesvirus pseudorabies virus (PRV). The immunological mechanisms giving protection against PRV are poorly understood. Passively or actively acquired serum antibodies as well as mucosal IgA antibodies only partially contribute to protection. Pigs that are immune after a first infection do not show a secondary B cell response upon reinfection. However, such immune pigs develop a strong secondary lymphoproliferation response upon reinfection (Kimman *et al.*, 1995*a*). This dichotomy between secondary B and T cell responses suggests that an effective proliferative T cell memory response prevents a secondary B cell response and may be responsible for quick elimination of challenge virus. The lymphoproliferative response may induce the emergence of cytolytic cells. However, other antiviral functions, such as the release of interferon-γ and tumour necrosis factor (Feduchi *et al.*, 1989; Lučin *et al.*, 1994) are probably associated with the lymphoproliferative response.

Knowledge on protective virus antigens is also incomplete. Previously, the PRV glycoproteins gB, gC and gD have been identified as targets for neutralizing antibodies (Ben-Porat *et al.*, 1986; Eloit *et al.*, 1988; Marchioli *et al.*, 1988). PRV gC has been identified as a target for cytotoxic T lymphocytes (CTLs) (Zuckermann *et al.*, 1990). PRV gB and gC also induced a lymphoproliferative response (Kimman *et al.*, 1995*a*). An important role for PRV gC in inducing protection has been further demonstrated; a gC-negative mutant was less effective in protecting mice against PRV than gE- or gG-negative mutants (Zuckermann *et al.*, 1990). Monoclonal antibodies (MAbs) directed against gB, gC and gD passively protected mice and pigs against a lethal PRV infection, whereas MAbs against gE protected only mice (Eloit *et al.*, 1988; Marchioli *et al.*, 1988; Van Oirschot *et al.*, 1988).

In this study we characterized porcine cytolytic cells that lysed PRV-infected target cells. Studies to examine the significance of CTLs against PRV-infected cells have previously been hampered by difficulties in measuring major histocompatibility complex (MHC)-restricted cytotoxicity in pigs. However, miniature pigs inbred for their MHC (Sachs *et al.*, 1976) have now become available, as well as an immortalized L14 B cell line of haplotype d/d (Kaeffer *et al.*, 1990). The reported suppression by PRV of cellular MHC class I expression (Mellencamp *et al.*, 1991) may also have hampered the detection of PRV-specific CTLs. In a previous report we established that the L14 cell line can be infected with PRV and can be lysed by lymphocytes from PRV-immune minipigs (Kimman *et al.*, 1995*b*). In addition, PRV infection did not down-regulate MHC expresssion on L14 cells.

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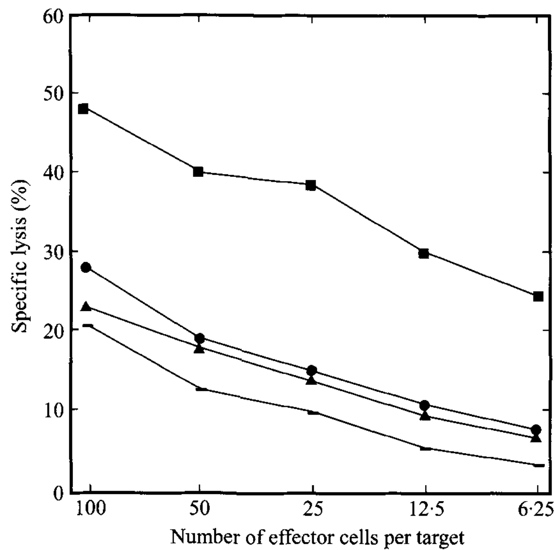
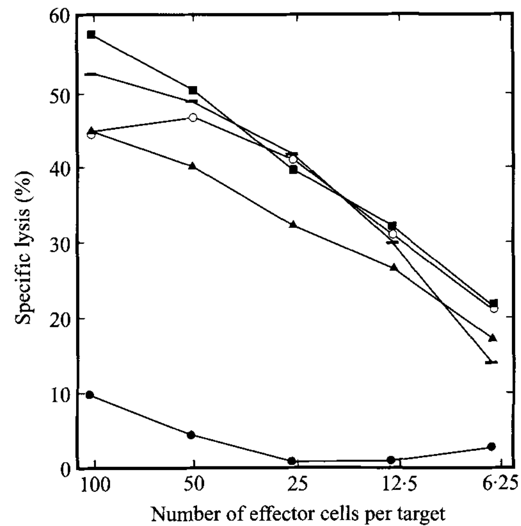


—■— Target L14–NIA3 ; immune minipig —●— Target L14 ; immune minipig

—▲— Target L14–NIA3 ; control minipig —— Target L14 ; control minipig

Fig. 1. Lysis of PRV-infected and uninfected target L14 cells. PBMCs from an immune and a non-immune SLA$^{d/d}$ minipig were stimulated *in vitro* with PRV for 6 days and incubated for 5 h with PRV strain NIA3-infected and uninfected ^{51}Cr-labelled target L14 cells. Lysis of the target cells was determined by measuring the release of ^{51}Cr in the supernatant after incubation. Each percentage is the mean of four replicates. One of eight duplicate experiments is shown.



Effector cell pre-treatment:

—■— None —— Anti-CD8 + complement

—●— Anti-CD2 + complement —○— Complement only

—▲— Anti-CD4 + complement

Fig. 2. Phenotype of the effector cell population. PBMCs from an immune SLA$^{d/d}$ minipig were stimulated *in vitro* with virus and treated with MAbs directed against porcine CD2, CD4 or CD8, and complement, prior to testing in a ^{51}Cr-release assay using infected L14 cells. Controls included pre-treatment of the cells with medium only or complement only. One of two duplicate experiments is shown.

To prepare cytolytic cells, we immunized miniature pigs homozygous for MHC (SLA$^{d/d}$) (Sachs *et al.*, 1976) with the PRV vaccine strain 783 (Moormann *et al.*, 1990) and subsequently with the virulent NIA-3 strain. At various intervals after infection, we obtained peripheral blood mononuclear cells (PBMCs) from immune and control animals and stimulated them *in vitro* with live PRV. The PBMCs obtained from immune SLA$^{d/d}$ minipigs and stimulated *in vitro* with PRV for 6 days efficiently lysed PRV-infected L14 cells in a 5 h cytolytic assay. Non-infected L14 cells were lysed less efficiently by the same effectors. In contrast, effector cells obtained from non-immune minipigs lysed infected and non-infected L14 cells only at background levels (Fig. 1). Characteristics of the PRV infection in L14 cells, the influence of PRV on MHC class I and II expression and the ^{51}Cr release assay we used have been described (Kimman *et al.*, 1995*b*).

In further experiments we characterized the effector cells responsible for the killing of PRV-infected L14 cells. First, to examine whether the observed cytolysis was MHC-restricted, we compared the cytolysis by effector cells obtained from immune minipigs (SLA$^{d/d}$ haplotype) and from immune outbred pigs (Dutch landrace). Effector cells from 11 different immune landrace pigs of different litters were approximately equally effective in lysing PRV-infected SLA$^{d/d}$ L14 cells as effector cells from inbred SLA$^{d/d}$ minipigs. Although the minipig d haplotype is similar to the H4 haplotype, which is present in landrace pigs (E. Hensen, personal communication), the high number of outbred pigs that gave similar results makes it very likely that the cytotoxicity was, at least to a large extent, not MHC-restricted. Second, depletion experiments were done to characterize the phenotype of the cytolytic cells. We generated effector cells by *in vitro* antigenic stimulation with virus and treated the effector cells before the cytolytic assay with MAbs directed against porcine CD2, CD4 or CD8, and complement, as described (Kimman *et al.*, 1993). Lysis was nearly completely abrogated by treatment of the effector cells with MAb directed against CD2 and complement, but not with MAbs directed against CD4 or CD8 and complement (Fig. 2). Because the CD8^{dull+} cell population (Pescovitz *et al.*, 1985; Saalmueller *et al.*, 1994) may not have been completely depleted by this treatment (Kimman *et al.*, 1993), the experiments indicated that the cytolysis is mediated by CD2$^+$CD4$^-$CD8$^-$ or CD2$^+$CD4$^-$CD8^{dull+} cells, but not by CD2$^+$CD4$^-$CD8$^{bright+}$ cells. Third, to corroborate further the finding that cytolysis is not mediated by classical CD8$^+$ MHC-restricted CTLs that use *de novo* synthesis of virus proteins and presentation of antigenic peptides by MHC class I molecules as the route of

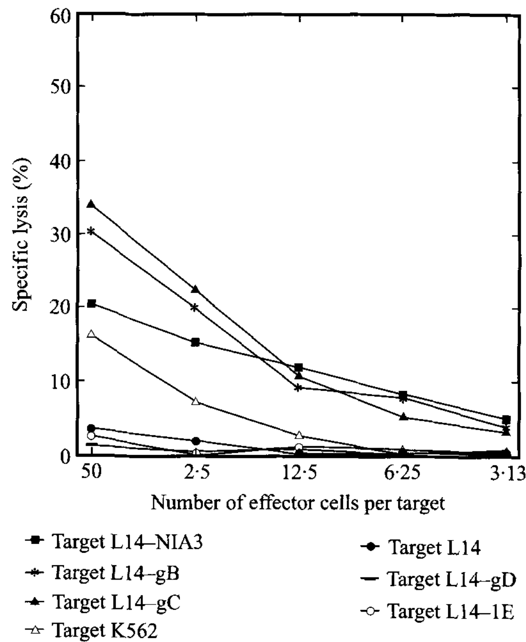


Fig. 3. Lysis of gB- and gC-transfected L14 cells. PBMCs from an immune SLA$^{d/d}$ minipig were stimulated *in vitro* with PRV and examined for cytolysis of gB-, gC-, gD- and IE-transfected L14 cells, PRV-infected and uninfected L14 cells and K562 cells. One of four duplicate experiments is shown.

antigen processing, we compared the capacity of *in vitro* antigenic stimulation with UV-killed PRV with that of live PRV to generate cytolytic effector cells. Inactivated antigen for *in vitro* restimulation was prepared by exposing part of a virus suspension to UV light for 10 min in Petri dishes (4000 $\mu W/cm^2$). Lack of infectious virus was subsequently checked by cell culture. Both treatments generated cytolytic effector cells. Fourth, because the experiments described so far suggested that cytolysis was mediated by non-MHC class I-restricted natural killer (NK)-like or lymphokine-activated killer (LAK) cells, we subsequently examined whether effector cells from immune and non-immune pigs also lysed the NK-susceptible target cell line K562. PBMCs, obtained from immune SLA$^{d/d}$ minipigs and immune outbred landrace pigs and stimulated *in vitro* with PRV, lysed K562 cells in addition to PRV-infected L14 cells. In contrast, this protocol failed to generate K562-lysing cells in non-immune animals (four experiments; data not shown). Thus, these experiments indicated the emergence of NK/LAK cells in PBMCs from immune animals upon *in vitro* antigenic stimulation with PRV. Fifth, to examine the significance of *in vitro* restimulation with virus antigen, we compared the cytolysis of PRV-infected L14 cells by unstimulated effector cells (i.e. PBMCs grown *in vitro* for 6 days without antigenic stimulation) and PRV-restimulated effector cells. *In vitro* restimulation with PRV augmented cytolysis approximately twofold at all effector/target cell ratios.

As an initial attempt to examine which virus structures are important in the cytolysis of PRV-infected cells by LAK cells, we transfected L14 cells with plasmid pEVhis14 containing one of the virus genes encoding gB, gC, gD or the immediate early (IE) protein, or with the empty control vector pEVhis14. L14 cell lines stably expressing gB, gC, gD or the IE protein were generated as described (Kimman *et al.*, 1995*a*). Flow cytometric analysis revealed that expression of gC on the transfected cell was high. In contrast, expression of gB, gD and the IE protein appeared low (Kimman *et al.*, 1995*a*). Effector cells generated by *in vitro* stimulation of PBMCs from immune animals with PRV [or by interleukin 2 (IL-2) stimulation] lysed gB- and gC-transfected cells approximately as efficiently as infected cells, in contrast to IE- or gD-transfected cells. Cells transfected with the empty pEVhis14 vector alone were lysed to the same degree as non-transfected and non-infected L14 cells (Fig. 3).

Finally, we examined whether LAK cells lysing PRV-infected target cells could also be generated by treatment of PBMCs for 6 days with 500 U/ml recombinant human IL-2 (Proleukin; Eurocetus). Stimulation with IL-2 generated cells lytic for PRV-infected L14 cells and K562 cells in both immune and non-immune animals.

Despite the MHC matching of target and effector cells, the bulk of cytolytic cells, as demonstrated in this study, displayed the characteristics of LAK cells: cytolysis appeared not to be MHC-restricted; the NK/LAK-susceptible cell line K562 was lysed; depletion experiments indicated that cytolysis was not mediated by classical $CD2^+CD4^-CD8^+$ CTLs; and finally, cytolysis was also induced by inactivated virus, indicating that *de novo* protein synthesis and the MHC class I pathway of antigen presentation to $CD8^+$ cells were not important in the generation of the cytotoxic response. Although the cytolytic cells were not restricted to virus-infected cells, the induction of cytolytic cells by *in vitro* restimulation with PRV only occurred in PRV-immune animals.

From these results two interesting points emerge. First, because the majority of cytolytic cells appeared to be LAK cells, the question arises as to whether MHC-restricted PRV-specific CTLs are important in the protection of pigs against PRV. Because our findings point to a potential role for LAK cells, we speculate that specific CTLs play a minor role in protective immunity or that the detection of these CTLs is severely hampered by LAK cells. Demonstrating MHC-restricted CTLs in the pig may require the cloning of cytolytic cells early after their induction. Furthermore, it may be that virus-specific MHC-restricted CTLs are important in clearing a primary infection but play a minor role in providing protection against reinfection. Second, the porcine species is unique in having high numbers of $CD4^-CD8^-$ 'double negative' and $CD4^+CD8^+$ 'double positive' T

cells, for which no functions have been described, in addition to the classical $CD4^+CD8^-$ T-helper cell and the $CD4^-CD8^+$ CTL. Moreover, CD8 expression is biphasic (Pescovitz *et al.*, 1985). The results of our depletion experiments indicate that cells with NK/LAK cell activity belong to the $CD2^+CD4^-CD8^-$ or $CD2^+CD4^-CD8^{dull+}$ subsets. Our results partly agree with those of Pescovitz *et al.* (1988), who also demonstrated CD2 on porcine NK cells, and CD8 on a substantial proportion of these cells. In addition, Saalmueller *et al.* (1994) showed that $CD5^-$ cells with dull CD8 expression had the functional characteristics of NK cells. Unfortunately, the lack of suitable reagents against the pig α/β and γ/δ T cell receptors (Lunney, 1993) makes it impossible to characterize these cytolytic cells further and to state conclusively whether the cytolytic cells demonstrated in this study belong to the T cell compartment.

Glycoprotein B- and gC-transfected cells were lysed as efficiently as PRV-infected cells, in contrast to IE- or gD-transfected cells. However, the level of expression of gD and the IE protein may have been too low. Our findings contribute to the existing and conflicting data regarding the requirements for virus protein expression in natural killing. While Bishop *et al.* (1986) reported that herpes simplex virus glycoproteins are the relevant structures recognized by NK cells, Fitzgerald-Bocarsly *et al.* (1991) reported that immediate early gene expression is sufficient to render cells susceptible to NK cells. Our data do not contradict either of these possibilities and do not imply that gB and gC are target structures recognized by NK/LAK cells.

Previously, several studies have demonstrated proliferative T cell responses in pigs upon immunization with PRV. We speculate that IL-2 and other lymphokines, secreted by PRV-sensitized T cells, increase NK/LAK cell-mediated cytolysis, which early after (re)infection may eliminate virus-infected cells. Further studies should indicate the *in vivo* significance of NK/LAK cells in the protection of pigs against PRV.

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