Protective immunity against bovine leukaemia virus (BLV) induced in carrier sheep by inoculation with a vaccinia virus–BLV env recombinant: association with cell-mediated immunity

Kazue Ohishi,† Hidemi Suzuki,† Toshiko Yamamoto,† Tadashi Maruyama,† Keizaburo Miki,† Yoji Ikawa,‡ Shigeru Numakunai,‡ Kosuke Okada,‡ Kan-ichi Ohshima‡ and Masanobu Sugimoto†

†Corporate Research and Development Laboratory, Tonen Corporation, 1-3-1 Nishi-Tsurugaoka, Ohi-machi, Iruma-gun, Saitama 354, ‡Tsukuba Life Science Center, The Institute of Physical and Chemical Research and ‡Department of Veterinary Pathology, Iwate University, Japan

The effects of vaccination of sheep with a recombinant vaccinia virus (rVV) expressing the bovine leukaemia virus (BLV) envelope glycoprotein (gp60) were studied by determining BLV titres in peripheral blood leukocytes after vaccination and challenge. The proliferation of BLV was suppressed markedly, not only when rVV was inoculated prior to challenge with BLV, but also when it was inoculated after challenge. These results indicate that vaccination with rVV induces protective immunity that can suppress the growth of BLV in carrier animals. Since rVV induced a strong anti-BLV delayed-type hypersensitivity response without producing detectable levels of binding or neutralizing antibodies, and there was no apparent correlation between the humoral immune response and BLV proliferation, a cell-mediated immune response was assumed to play a major role in protective immunity.

Introduction

Bovine leukaemia virus (BLV) is the aetiological agent of enzootic bovine leukaemia; the nucleotide sequence and gene organization of its genome are similar to those of human T cell leukaemia virus types I and II (HTLV-I and -II) (Seiki et al., 1983; Sagata et al., 1984, 1985; Shimotohno et al., 1985). Since BLV induces leukaemia in sheep at a high incidence within a relatively short period of time (Olson & Baumgartener, 1976; Kenyon et al., 1981), this animal system provides a good disease model for both BLV and HTLV infection.

A relatively long incubation period exists before the onset of disease after infection with HTLV-I and -II, and human immunodeficiency virus (HIV). Therefore, it is likely that the host immune response against these viruses is crucial in determining the rate of disease development.

We have undertaken a series of experiments in sheep using a recombinant vaccinia virus (rVV) expressing the BLV envelope glycoprotein (gp60) as an experimental probe. Vaccination with rVV was done either prior to or after challenge with BLV, and the BLV titre in peripheral blood leukocytes (PBLs) was determined to evaluate the effect of the vaccination. Simultaneous assessments of humoral and cell-mediated immune responses were carried out. The experiments indicate that vaccination with rVV suppresses the proliferation of BLV in carrier animals, presumably by augmenting host cell-mediated immune responses.

Methods

Animals. Male sheep (3 to 4 years old) were used for the vaccination and challenge experiments in Expt. I, 8-month-old male and female sheep were used for the study of the delayed-type hypersensitivity (DTH) response induced by rVV inoculation, as well as for the vaccination and challenge experiments in Expt. II, and 8-month-old male animals were used in Expt. III. All the animals vaccinated with rVV were kept in a pen furnished with isolated drainage, and their excretions were collected and sterilized with an autoclave or disinfectant. All experiments were conducted according to the guidelines of The Ministry of Education, Science and Culture, Japan.

All animals were serologically BLV-negative before the experiments. A cow known to have been BLV-positive since 1984 was used as a donor of BLV-infected PBLs for challenge; 2500 to 5000 syncytia/10⁶ PBLs were formed. In situ hybridization indicated that about 10% of all PBLs from this cow were BLV-positive.

Viruses and cells. We used an rVV (mO-HA/ATI) expressing the BLV envelope glycoprotein under the control of a promoter of the A-type inclusion body protein gene of cowpox virus (Funahashi et al., 1988), and an attenuated vaccinia virus strain, LC16mO (kindly donated by the Chiba Serum Institute), as a vector (Hashizume et al., 1985). The construction of rVV and its immunogenicity in rabbits have been reported (Ohishi et al., 1990). Rabbit kidney cells (RK13) were grown at 37 °C in Eagle's MEM supplemented with 10% calf serum.
and used for the growth of rVV and LC16mO. Ovine embryonic cells (KTO-1) (kindly donated by Dr H. Koyama, Kitasato University, Japan) were cultured in Dulbecco's MEM (DMEM) supplemented and used for the growth of rVV and LC16mO. Ovine embryonic cells (KTO-1) (kindly donated by Dr H. Koyama, Kitasato University, Japan) were cultured in Dulbecco's MEM (DMEM) supplemented with 10% foetal calf serum and were used as indicator cells in the syncytium formation assay.

**Isolation of PBLs.** Briefly, buffy coat cells were obtained by centrifuging heparinized blood at 1600 g for 20 min at 4 °C. The PBL fraction was obtained by centrifuging buffy coat cells in 60% Percoll solution at 1600 g for 20 min at room temperature.

**Syncytium formation assay.** The assay was performed according to the procedure described by Itohara & Mizuno (1984). Ovine embryonic cells (10^4) were grown for 24 h in a 35 mm dish with 2 mm grids and inoculated with 10^6 BLV-infected ovine PBLs in DMEM containing 1% DMSO and 4 μg/ml polybrene. Cells were cultured for 24 h. PBLs were removed and culturing was continued for another 5 days, after which the cells were fixed with 2% paraformamide solution, and stained with May-Gruenwald and Giemsa stain. Cells containing more than five nuclei were counted as syncytia. There was a roughly linear relationship between the number of syncytia formed and doses of PBLs between 10^5 and 10^6 cells (data not shown). To confirm the reproducibility of the assay system, we assessed the number of syncytia formed by an aliquot of a standard BLV suspension obtained from the supernatant of a culture of BLV-producing foetal lamb kidney cells in Expt. II and III; the number of syncytia formed was relatively constant (between 2430 and 3410 in Expt. II, and 2030 and 2620 in Expt. III).

**Determination of neutralizing antibody titre by a syncytium inhibition assay.** The assay was performed as described above except that sample serum (2% final concentration) was added, allowing its neutralizing activity to be determined. When bovine anti-BLV serum and BLV-infected cells were added to this system, syncytium formation was inhibited completely (data not shown).

**ELISA.** Titration of anti-BLV antibodies was carried out using a standard ELISA method. Briefly, BLV virions in 0.1% Triton X-100 were adsorbed to each well of a 96-well plate. After coating with 0.5% casein in PBS, ovine serum diluted 1/100 was added to each well and incubated at 37 °C for 1 h; peroxidase-conjugated donkey anti-sheep IgG or IgM was used as a secondary antibody. The absorbance at 492 nm was read using an automatic ELISA reader.

**Immunodiffusion.** This was carried out using partially purified BLV virions as the antigen and sera from cattle with leukaemia as a positive control, both from a commercial kit obtained from the Kitasato Institute, Tokyo, Japan.

**DTH responses of sheep vaccinated with rVV.** Sheep were vaccinated with 10^8 p.f.u. rVV, or with parental VV LC16mO as a control, and 7 days after vaccination they were challenged intradermally with BLV (2 units/0.1 ml, as determined using the immunodiffusion kit) or keyhole limpet haemocyanin (KLH) (0.5 μg/0.1 ml) as a control. Skin biopsy was carried out 3, 24, 48 and 72 h after challenge, and biopsies were fixed with 10% formalin and embedded in paraffin; thin sections were stained with haematoxylin and eosin, and observed under light microscopy.

**Results**

**Determination of challenge dose**

BLV-infected bovine PBLs (8 × 10^2, 8 × 10^3 or 8 × 10^4) were inoculated subcutaneously into groups of two sheep. Infection of sheep PBLs with BLV was assessed using a syncytium formation assay, as well as by an immunodiffusion test. All sheep were BLV-positive in one of the tests by day 60 (data not shown), indicating that 8 × 10^2 BLV-infected bovine PBLs could infect sheep. Based on these results, we inoculated sheep with 10^5 PBLs in Expt. I and III, and 2 × 10^3 PBLs in Expt. II.

**Effects of vaccination with rVV on the growth of BLV in naive sheep challenged with a large dose of BLV-infected PBLs**

In Expt. I sheep were divided into two groups: group A (four sheep) was vaccinated intradermally with 10^8 p.f.u. rVV; group B (five sheep) was inoculated with 10^8 p.f.u. LC16mO. At 20 weeks post-inoculation (p.i.), all sheep were subcutaneously challenged with 10^5 bovine PBLs. Syncytia were detected in all sheep in group B 3 weeks post-challenge (p.c.) (Fig. 1b); these values reached plateau levels at about 6 weeks. In contrast, the number of syncytia detected in animals in group A (Fig. 1a) was very low; the number of syncytia formed per 10^6 PBLs detected at 26 weeks p.c. was between 12 and 17, whereas that in animals in group B was between 60 and 340. The antibody response was monitored simultaneously. Fig. 1(c and d) shows the anti-BLV IgG response assessed by ELISA. Vaccination with rVV alone did not induce a detectable antibody response. Challenge with
BLV-infected bovine PBLs caused the antibody titre in animals in group A to increase sharply within 3 weeks p.c., whereas those of the controls (group B) increased rather slowly. Thus, animals in group A showed a maximal antibody response more quickly than those in the control group, although the responses at 26 weeks p.c. did not differ very much, suggesting that rVV inoculation stimulated helper T cells without inducing detectable antibodies. One animal (no. 19) which had a remarkably high titre of BLV had antibody titres similar to those of the other animals. Anti-BLV IgM responses were not observed in either group during the experimental period. A significant neutralizing antibody titre, assessed using the syncytium inhibition assay, was not detected in any serum 20 weeks after vaccination with rVV (syncytium inhibition was 20.1% for group A and 18.7% for the control group). The titre was also negligible 9 weeks p.c. (25.8% for group A, 31.8% for controls). Thus, there was no apparent correlation between the humoral antibody response and the proliferation of BLV in PBLs.

Effects of rVV vaccination on the growth of BLV in naive sheep challenged with a smaller dose of BLV-infected PBLs

In Expt. II, six sheep were inoculated with rVV (group C) and six sheep were inoculated with LC16mO (group D); 9 weeks later they were challenged with $2 \times 10^3$ BLV-infected PBLs. As shown in Fig. 2(a and b), no syncytia were detected in two animals in group C, or one animal in group D during the experimental period. Although syncytia were detected in four sheep in group C, the number was relatively small. As shown in Fig. 2(c and d), inoculation with rVV did not induce detectable anti-BLV antibodies, as was the case in Expt. I. Challenge with BLV-infected PBLs produced antibody responses of the secondary type in animals infected with BLV in group C, whereas those of the primary type were observed for those in group D.

Effects of rVV inoculation on the growth of BLV in BLV-carrier sheep

In Expt. III, the effect of vaccination with rVV on sheep carrying BLV was investigated. Ten sheep were challenged with $10^5$ BLV-infected PBLs, and the number of PBL syncytia was determined 5 weeks later. These sheep were then divided into two groups (E and F), group E was inoculated with rVV, whereas group F was inoculated with LC16mO. As shown in Fig. 3(a and b), syncytium formation in three group F animals continued to increase, whereas that in group E animals increased only slightly or decreased. The effect of the vaccine could not be determined for two animals in each group because the number of syncytia formed was too low. After challenge with BLV-infected PBLs, anti-BLV antibody titres increased in all animals of both groups, although
the variation from animal to animal was relatively large (Fig. 3c and d) and there was no significant difference between the two groups. Vaccination of animals carrying BLV with rVV did not induce a secondary antibody response. These results suggest that antibodies directed against the BLV envelope glycoprotein did not contribute to the suppression of BLV growth.

DTH skin reactions of sheep vaccinated with rVV

Although the induced humoral immune response was poor, vaccination with rVV did induce a marked DTH response. Two sheep were inoculated with rVV and challenged intradermally with BLV virions 7 days later; strong DTH skin reactions were observed. This reaction was not seen in two animals vaccinated with LC16mO. These clinical results were confirmed by histological examination (Fig. 4): skin lesions of animals vaccinated with rVV showed intensive infiltration by mononuclear cells, but poor infiltration by polymorphonuclear leukocytes 48 and 72 h p.c., and no infiltration was apparent 3 h p.c. This result is consistent with the fact that anti-gp60 antibodies were virtually absent from the animals. The DTH skin reactions were highly antigen-specific; no reaction was observed when animals vaccinated with LC16mO were challenged with BLV virions, nor when animals vaccinated with rVV were challenged with KLH.

Discussion

This study has demonstrated that vaccination with rVV expressing BLV gp60 induces protective immunity, suppressing the growth of BLV in carrier animals. First, although the inoculation of naive sheep with rVV does not protect the animals completely against BLV challenge, it does induce protective immunity in the resultant carrier animals, consistently suppressing the proliferation of BLV over a relatively long time period. Second, vaccination with rVV is effective in sheep which already carry BLV.

Humoral immunity seems unlikely to play a major role in the protective immunity induced by vaccination with rVV because (i) rVV inoculation itself does not induce a detectable antibody response, (ii) rVV inoculation of animals carrying BLV suppresses the growth of BLV with virtually no effect on the antibody response and (iii) antibody titres do not correlate with the degree of BLV proliferation in PBLs.

Unexpectedly, rVV itself was not able to induce detectable anti-BLV antibodies in sheep, despite the fact that the same rVV has been shown to be immunogenic in rabbits (Ohishi et al., 1990). This failure may be ascribed to poor expression of gp60, probably due to the low proliferative capability of rVV in sheep skin; the skin lesion at the site of inoculation was much smaller in sheep than it was in rabbits (unpublished observation). Portetelle et al. (1991) have reported recently that sheep inoculated with rVV expressing the BLV envelope glycoprotein produce neutralizing antibodies to BLV. This discrepancy may be because they used the Copenhagen strain of VV. It is generally accepted that a relatively large amount of antigen is necessary for the induction of the humoral immune response, although much less antigen is sufficient to induce T cell-mediated immunity, including helper T cell and DTH T cell responses (Ohishi et al., 1988). We have shown that inoculation of sheep with rVV induces a strong DTH response and stimulates helper T cells, as deduced from the memory effect for the antibody response. This fact strongly suggests that the cell-mediated immune response plays a major role in the suppression of BLV growth in carrier animals. The mononuclear cells which infiltrated the skin lesions in the DTH response were about 65% CD8+ and 25% CD4+ 48 h after challenge (K. Okada, S. Numakunai & K. Ohshima, personal communication), suggesting that CD8+ cytotoxic T lymphocytes suppress BLV proliferation by killing BLV-infected cells. Interestingly, Doherty et al. (1990) argue that CD8+ T cells play a major role in the DTH reaction to lymphocytic choriomeningitis (LCM) in mice infected with LCM virus. It is also possible that CD4+ T cells are involved in the suppression of the growth of BLV by releasing lymphokines such as interferon γ. Some reports do support a role for cell-mediated immunity in retrovirus infection in vivo, e.g. Earl et al. (1986) have reported circumstantial evidence that a cell-mediated
immune response is involved in the protection of mice against Friend leukaemia virus (FLV), as determined by vaccination with an rVV expressing the FLV envelope glycoprotein.

The question arises of which cells are the putative target of the cell-mediated immune response because BLV-infected cells do not usually express gp60 on their cell surface. We believe that some BLV-infected cells in carrier animals could express the antigen in an unidentified fashion because antibodies against BLV gp60 remain at relatively high levels throughout the life-span of BLV-infected animals once induced.

The fact that the level of proliferation of BLV varies greatly between individual animals suggests that the host immune response differs. We believe that this individual variability should not be ascribed to experimental failure because the titres of BLV and antibodies in an animal showed consistent patterns during the experimental period. Animals which had not been vaccinated with rVV but which had very low BLV titres during the experimental period may have an immune response suppressing the proliferation of BLV similar to that in animals vaccinated with rVV. The variability in the immune response to BLV gp60 may be explained by some feature of the immune response (Ir) gene of the major histocompatibility complex. For instance, Miyazawa et al. (1988) have reported that proliferative murine T cell responses to the FLV envelope glycoprotein are highly dependent on the haplotype of the Ir gene. Takahashi et al. (1988) have also reported that murine cytotoxic T lymphocyte responses to the HIV envelope glycoprotein are dependent on the H-2 haplotype.

BLV is integrated into the host genome as proviral DNA, so antibodies mainly effective against free virus would be unlikely to eliminate the virus from carrier animals, although they can destroy virus-infected cells by antibody-dependent cytotoxicity reactions. Miller and colleagues (Miller & Van der Maaten, 1978; Miller et al., 1983; Miller, 1986) first demonstrated that vaccination with BLV virions together with adjuvant partly protected animals against BLV infection. The importance of the antibody response in protection against BLV infection has been demonstrated further by other investigators (Onuma et al., 1984; Kono et al., 1986). For complete protection against BLV, a vaccine must induce strong immune responses, both humoral and cell-mediated. It still remains to be determined whether other viral antigens, such as core proteins, can induce protective immunity. The results showing that vaccination with rVV is effective even in carrier animals have very important implications for the prevention of the development of disease in retrovirus carriers in general, e.g. carriers of HIV.

References


SAGATA, N., YASUNAGA, T., TSUZUKI, K. J., OHISHI, K., OGAWA, Y. & IKAWA, Y. (1985). Complete nucleotide sequence of the genome of


(Received 3 December 1990; Accepted 18 April 1991)