T cell proliferative response to bovine leukaemia virus (BLV): identification of T cell epitopes on the major core protein (p24) in BLV-infected cattle with normal haematological values

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Peripheral blood mononuclear cells (PBMCs) from bovine leukaemia virus (BLV)-seronegative cattle and from BLV-seropositive cows either with normal haematological values or persistent lymphocytosis were tested for their proliferative response to BLV antigens. Cells from only BLV-infected cattle with normal lymphocyte counts were stimulated to a detectable level by the fetal lamb kidney cell supernatant containing BLV antigens. Proliferation assays performed with the purified major core protein p24 indicated that this protein has to be processed through a chloroquine-sensitive compartment before being recognized by CD4+ T lymphocytes. Forty-one 15-mer overlapping peptides spanning the entire p24 sequence were synthesized and analysed for their stimulating potential. It appeared that two regions included T cell epitopes recognized by PBMCs from three of five animals tested. These regions were represented by amino acids 31 to 55 (PGSQVWIQTL-RLAILQADPTPADLE) and 141 to 165 (AESYVE-FVNRLQISLADNLPDGVPK). The possible implication of this cell-mediated immune response in BLV pathogenesis and vaccine development is discussed.

Introduction

Bovine leukaemia virus (BLV) is the aetiological agent of the enzootic bovine leukosis, a lymphoproliferative disease characterized initially by the presence of anti-BLV antibodies. In some cases, animals can develop persistent lymphocytosis (PL) and/or lymphoid tumours (for reviews see: Burny et al., 1987; Van der Maaten & Miller, 1990).

This B lymphocytotropic (Paul et al., 1977; Levy et al., 1987), trans-activating (Rosen et al., 1986; Derse, 1987, 1988) retrovirus induces an antibody response mainly against its two major structural proteins: the external membrane glycoprotein gp51 and the major core protein p24 (Miller & Van der Maaten, 1976; Bex et al., 1979; Meirom et al., 1985). These antibodies may persist throughout the animals' life and even increase as the disease progresses toward PL and/or tumours.

Until now, low viral expression, if any, has been detected in freshly isolated peripheral blood mononuclear cells (PBMCs) of cattle (Jensen et al., 1991). This phenomenon is related to a transcriptional blocking of the integrated provirus (Kettmann et al., 1980, 1982). Nevertheless, the persistence of an anti-BLV humoral response suggests a continuous antigenic stimulation in vivo. As these antibodies are neither capable of eliminating the virus nor protecting animals from the progression of disease, an efficient control of the virus infection could involve the cellular arm of the immune system as shown for other viruses. Indeed many viral proteins have been identified as targets for the recognition by antigen-specific helper or cytotoxic T lymphocytes (CTLs) (Gotch et al., 1987; B. D. Walker et al., 1987; McGraw et al., 1990; Levely et al., 1991). In several cases, immunodominant T cell epitopes have been mapped using the synthetic peptide approach (Ertl et al., 1989; Ho et al., 1990; Wallace et al., 1991; Kutubuddin et al., 1992; Ou et al., 1992). In particular, some helper T cell determinants on BLV gp51 have been identified using PBMCs from BLV-infected cattle with normal lymphocyte counts (Callebaut et al., 1993).

The aim of this study was to investigate the cellular immune response to BLV antigens in infected cattle. To control a possible background stimulation caused by in vitro virus expression after short-term lymphocyte culture (Stock & Ferrer, 1972; Baliga & Ferrer, 1977; Driscoll et al., 1977), we used the proliferation assay described by Callebaut et al. (1993). This assay allowed us to demonstrate the existence of a specific CD4+ T lymphocyte-mediated response to BLV and its major core protein p24 in BLV-infected cows with normal haematological values. Furthermore we identified several
T cell epitopes of p24 using a set of overlapping synthetic peptides spanning the entire protein sequence.

Methods

Animals. This study included nine BLV-seropositive cows characterized as such by their anti-gp51 antibody titres determined by ELISA (Portetelle et al., 1989). Cows B68, B71, B73, B79 and B80 had normal lymphocyte counts and animals B70, B76, B77 and B85163 were classified as being in PL, based on the EC-leukosis key (Mammerickx et al., 1978). Three BLV-seronegative field animals (B78, B10 and B11) served as control blood donors.

Monoclonal antibodies (MAbs). MAbs BW5C6H11 and BWD12G8 specific for BLV p24 protein were kindly supplied by Drs P. Coppe and A. Collard, CER, Marloie, Belgium. MAb ILA55 (ascitic fluid), recognizing bovine B cells, was a gift from Dr J. Naessens, ILRAD, Kenya. MAbs CC8 (anti-BoCD4) and CC63 (anti-BoCD8) (both ascitic fluids) were kindly provided by Dr C. Howard, Compton Laboratory, Newbury, U.K. MAb 8C11 (anti-BoCD5) was produced in our laboratory.

Viral antigens. BLV antigens were obtained from a constitutively infected fetal lamb kidney cell line (FLK). FLK cells were grown to confluence in Opti-MEM (Gibco) supplemented with 2 mm-glutamin (Gibco), 50 μg/ml gentamicin (Gibco) and 2.5% heat-inactivated fetal calf serum (FCS) (Gibco). The culture supernatant was then removed and FCS-free medium was added. Two to 3 days later, cells and supernatant were harvested and submitted to four freeze-thawing cycles which provided sufficient amounts of soluble p24 as demonstrated by a p24-specific capture ELISA (data not shown). This preparation was then centrifuged to eliminate cell debris and the resultant supernatant was stored at −20 °C. This supernatant was used as the antigen preparation for proliferation assays and further protein purification, and is referred to below as ‘FLK supernatant’.

To purify the p24 protein, this FLK supernatant was concentrated on a Miniant (Millipore) (membranes with a cut-off limit of 10K). Purification was performed by affinity chromatography using MAb BW5C6H11 coupled to Sepharose-4B beads (Pharmacia). Briefly, after passing the concentrated FLK supernatant through a Sepharose 4B column to preclear the sample, it was applied to the specific anti-p24 column. Following extensive washing with PBS pH 7.4, the bound antigen was desorbed by 0.1 m-glycine–HCl pH 2.8, immediately neutralized with 1 m-Tris–HCl pH 8 and dialysed against PBS. Residual contaminants were eliminated by gel filtration using a Superdex HR75 hi-load column (Pharmacia) in a FPLC system (Pharmacia). The recovered p24 was stored at −20 °C until use.

Purity was determined by SDS–PAGE (Laemmli, 1970) and silver staining (Merril et al., 1981) and specificity was ascertained by Western blot analysis using MAb BW1D2G8.

Synthetic peptides. Forty-one 15-mer overlapping peptides (by 10 amino acids), spanning the entire p24 sequence were synthesized by Chiron Mimotopes, based on the sequence data published by Rice et al. (1985). This sequence was considered convenient as the authors mentioned that the gag DNA sequence of the FLK–BLV provirus differed by only 3.5% from that of their published sequence obtained from a bovine tumour-derived BLV clone. The lyophilized peptides were resuspended in PBS pH 7.4 to form stock solutions (1 mg/ml), aliquoted and stored at −20 °C.

Isolation of PBMCs. Blood was collected by jugular venipuncture in a one-tenth volume of PBS buffer–1.5% EDTA solution and centrifuged at 1500 g for 15 min. The buffy coat was removed, diluted 1:2 in PBS pH 7.4, layered over a Ficoll-Hypaque gradient (density 1.077) and centrifuged at 1000 g for 25 min. The mononuclear cell layer was collected from the interface and washed three to four times in PBS (200 g for 10 min). Cells were suspended in FCS containing 10% DMSO and stored under liquid nitrogen until required.

Cell depletion. PBMCs (10 x 10^6/ml) were incubated with MAbs (ILA55, CC8 or CC63) diluted 1:1000 in PBS for 30 min at 4 °C. After washing in PBS, cells were suspended at 10 x 10^6/ml in RPMI 1640 medium supplemented with 25 mm-HEPES (Gibco), 2 mm-glutamin (Gibco), 1 mm-sodium pyruvate (Gibco), 5 x 10^-5 m-mercaptoethanol (Gibco) and 50 μg/ml gentamicin (Gibco) containing 10% rabbit complement (Sera-Lab) and incubated for 1 h at 37 °C. After three washes in PBS, cells were placed in RPMI medium containing 10% heat-inactivated autologous serum (autologous medium). Cell depletion was monitored by fluorescence-activated cell sorting analysis. B lymphocyte depletion of PBMCs from animals with normal haematological values always resulted in < 5% contaminating B cells. In contrast, in PL cows the remaining B lymphocytes could not be reduced to < 15 to 30% of the responder cell population. CD4+ and CD8+ T cell enrichment of the responder cells was accomplished by < 3% CD8+ and < 5% CD4+ contaminating T lymphocytes, respectively.

Lymphocyte proliferation assay. Antigen-presenting cells (APCs) were prepared by treating 10 x 10^6 PBMC/ml RPMI medium with 25 μg/ml mitomycin C (Sigma) for 1 h at 37 °C. After three washes in PBS, cells were suspended in autologous medium.

Responder cells were represented by B lymphocyte-depleted PBMCs. The CD4+- and CD8+-enriched populations were obtained after depletion of PBMCs from B cells and CD8+ T lymphocytes or from B cells and CD4+ T lymphocytes respectively.

APCs (2 x 10^6) and responder cells (10^6) were mixed with or without (control culture) antigens in round-bottomed 96-well microplates (Nunc) in a total volume of 200 μl autologous medium. The plates were incubated in a 5% CO2 humidified atmosphere at 37 °C. To analyse the processing requirements, 2 x 10^6 APC/ml autologous medium containing the antigen were incubated in the absence or presence of a 100 μM-chloroquine solution (Sigma) in a 5% CO2 incubator at 37 °C for 24 h. The cells were washed extensively in medium before being seeded together with the responder cells in round-bottomed microplates in 200 μl autologous medium.

After 6 to 7 days of culture, each well was pulsed with 0.8 μCi [3H]methy1-thymidine (Amersham) in 40 μl RPMI medium for the last 18 h of the culture. The cells were collected using a cell harvester (Titertek 550) and incorporation of radioactivity into the DNA was measured by liquid scintillation counting (1205 Betaplate, Wallac, Pharmacia). All tests were set up as triplicate cultures (at least) and realized at least twice. Results were expressed either as c.p.m. ± S.D. or as stimulation index (SI) calculated by dividing the mean c.p.m. of stimulated cultures by the mean c.p.m. of control cultures. Data were analysed statistically by the Student’s t-test with P < 0.05.

Results

Proliferation of PBMCs in response to BLV antigens

Preliminary experiments carried out by standard proliferation assays (total PBMC, culture medium containing FCS) did not reveal any significant proliferative response to FLK supernatant (data not shown). We then used the modified proliferation assay described by Callerbaut et al. (1993) to analyse the antigen-specific response in BLV-infected cattle. APCs and responder
Fig. 1. T cell proliferative response of BLV-seronegative (B78, B10 and B11) (a), BLV-seropositive cows with normal lymphocyte counts (B68, B71, B73, B79 and B80) (b) and PL cattle (B70, B76, B77 and B85163) (c) to FLK supernatant (1:32 dilution) after 7 days of culture. Results are expressed as mean c.p.m. ± s.e.m. from quintuplicate cultures. Numbers above columns indicate SI. An asterisk denotes a statistically significant difference between stimulated cultures (containing antigen, ⨿) and control cultures (medium alone, □).

Proliferation of PBMCs in response to the p24 major core protein

To demonstrate the specificity of the proliferative response of PBMCs from infected animals with normal haematological values to the FLK supernatants, we purified the p24 major viral core protein from the FLK supernatant by affinity chromatography and gel filtration. Purity was analysed by SDS–PAGE and silver staining (Fig. 2). Four bands were visible, the strongest observed at 24K M₉. Specificity was ascertained by Western blotting and immunoenzymatic detection which revealed that all four bands were recognized by an anti-p24 MAb (BW1D2G8) and not by an irrelevant MAb specific for BoCD5 (8C11) (data not shown). This pattern of multiple bands could be related to the storage of the antigen at −20 °C (P. J. Walker et al., 1987).

The purified p24 was tested in a proliferation assay. Fig. 3 shows representative results from one non-infected cell line.
cow (B78) and two BLV-seropositive animals (B73 and B79). T cells from cow B78 did not proliferate in response to p24. In contrast, cells from cows B73 and B79 were specifically activated by this protein. The stimulation displayed a dose-dependent profile and once more, individual differences were observed. Animal B73 showed significant proliferation only at a protein concentration of 10 μg/ml, whereas cells from animal B79 were triggered at 10, 5 and even 1 μg/ml. Similar results were obtained with PBMCs from animals B68, B71 and B80 (data not shown).

These data proved the existence of p24-specific T lymphocytes in the peripheral blood of BLV-infected cattle with normal lymphocyte counts.

**Analysis of the T cell subset involved in antigen-specific proliferation**

CD4⁺- and CD8⁺-enriched T lymphocyte responder populations were obtained by complement-dependent cytotoxicity after treatment of PBMCs with anti-B cells and anti-CD8 or anti-B cells and anti-CD4 MAbs, respectively.

Antigen-specific proliferation assays were carried out by incubation of APCs with CD4⁺- or CD8⁺-enriched T cells in the presence of FLK supernatant (1:32 dilution) or purified p24 (1 μg/ml). Fig. 4 shows the results obtained from animals B79 and B80. It clearly shows that the population stimulated by FLK supernatant (B79 and B80) (Fig. 4a, b) and by p24 (B80) (Fig. 4b) displayed the CD4⁺ phenotype. No significant proliferative response was detected for CD8⁺-enriched T cells. CD4⁺-enriched T cell populations showed mostly higher SI values in response to FLK supernatant and p24 than the total responder T cells. The SI calculated from the data for total T cells of animal B79 stimulated with FLK supernatant was 7.0, whereas for CD4⁺ T cells, the corresponding SI was 8.2. Proliferation of total T cells from animal B80 incubated with FLK supernatant and p24 reached SI values of 4.7 and 4.5 respectively, in contrast to the results obtained for CD4⁺ responder T cells with corresponding SI values of 17.4 and 6, respectively. Analogous results were obtained with cells from animals B71 and B73 (data not shown).

Thus we showed that the p24-specific responder population in infected cattle with normal lymphocyte counts included cells belonging to the CD4⁺ T cell subset.

**Analysis of the processing requirements for p24**

CD4⁺ T cell activation is generally induced by a major histocompatibility complex (MHC) class II-positive APC after processing of an exogenous antigen through an intracytoplasmic acidic compartment (Berzofsky et al.,
1988). To test this hypothesis in our BLV system, APCs were incubated with p24 (10 μg/ml) in the absence or presence of chloroquine (100 μM), a lysosomotropic drug that raises the pH of this subcellular compartment. Twenty-four hours later, cells were washed and cultured with the responder T cells for 7 days. Results are expressed as mean c.p.m. ± s.d. from quintuplicate cultures. An asterisk denotes a statistically significant difference between stimulated cultures (containing antigen) and control cultures (medium alone).

Therefore it appeared that in our stimulation system the p24 protein required processing by APC through a chloroquine-sensitive compartment before becoming able to induce an antigen-specific activation of T cells.

**Proliferation of PBMCs in response to overlapping synthetic peptides of p24**

To localize T cell epitopes on the p24 protein recognized by PBMCs from infected cows with normal lymphocyte counts, we performed a proliferation assay including 41 overlapping synthetic peptides spanning the entire sequence of the p24 protein. Fig. 6 represents the peptide sequences shown to contain T cell epitopes. Cultures were set up by incubating APCs and responder cells for 6 days in medium alone (RPMI control), with FLK supernatant (1:32 dilution), purified p24 (10 μg/ml) or synthetic peptides (30 μg/ml). As indicated in Fig. 7, cells from the BLV-seronegative cow B78 (control) (Fig. 7a) did not show any proliferative response to the antigens tested. In contrast, cells from infected cattle B68 (Fig. 7b), B73 (Fig. 7d) and B80 (Fig. 7e) were stimulated by the control antigens (FLK supernatant and p24) and by different peptides. Thus, cells from animal B68 (Fig. 7b) proliferated after incubation with peptides 7 (31–45), 8 (36–50), 9 (41–55), 29 (141–155); cells from animal B73 (Fig. 7d) were stimulated by peptides 30 (146–160), 31 (151–165) and those from animal B80 (Fig. 7e) by peptides 7 (31–45) and 8 (36–50). At the time of the test, cells from animals B71 (Fig. 7c) and B79 (data not shown) were stimulated by the FLK supernatant only and did not proliferate in response to either purified p24 or any of the synthetic peptides tested. These screening assays were also performed with peptides used at a concentration of 5 μg/ml but no other stimulating peptides were detected.

To reveal eventual individual differences in the proliferating capacity of PBMCs after stimulation with a given peptide, we tried to estimate the dose-response curve. Fig. 8 represents the proliferative responses of cells from animals B68 (Fig. 8a) and B73 (Fig. 8b) stimulated with peptides covering a range of concentrations from 0.01 to 50 μg/ml. Animal B68 (Fig. 8a) was significantly stimulated by peptide 8 (36–50) at concentrations of 30 to 10 and 1 μg/ml, whereas peptide 29 (141–155) was effective only at 30 μg/ml. A roughly different profile was displayed by cells from animal B73 (Fig. 8b) which proliferated in response to peptides 30 (146–160) and 31 (151–165) at all concentrations tested (50, 30, 10, 1, 0.1 and 0.01 μg/ml).

We concluded that synthetic peptides representing different sequences of the p24 protein were able to stimulate T cells from BLV-infected cattle with normal lymphocyte counts. Individual differences in the nature of the sequence recognized by the T cells and the peptide concentration required for stimulation were observed.
Discussion

Using the modified proliferation assay described by Callebaut et al. (1993), we demonstrated the existence of activated virus-specific CD4+ T lymphocytes in the peripheral blood of BLV-infected cows with normal haematological values and identified several T cell epitopes on the p24 core protein using overlapping synthetic peptides.

Proliferation assays performed with the FLK supernatant containing BLV proteins suggested the existence of BLV-specific T cells in PBMCs from infected cattle with normal lymphocyte counts. In BLV-seronegative cows or in animals with PL such proliferation was not
shown. One possible explanation for the failure to detect an antigenic stimulation in PL cattle could be the relative inefficiency of B lymphocyte depletion in these cows (15 to 30% B cells could still be detected in the responder cell population), in which up to 80% of the PBMCs are surface immunoglobulin-positive cells. The remaining B cells and the subsequent potential viral expression would be responsible for the higher background proliferation impairing the detection of antigen-specific T cells (Fig. 1c).

Further analysis of this proliferative response revealed that the p24 major internal core protein constituted a target antigen for T lymphocytes and that the CD4\(^+\) T cell subset was involved. However our assays did not allow us to draw conclusions about the effector function of these cells.

Using overlapping synthetic peptides spanning the entire sequence of the p24 protein, we identified several T cell epitopes recognized by PBMCs of BLV-infected cows with normal lymphocyte counts. These assays revealed that PBMCs from animals B71 (Fig. 7c) and B79 (data not shown) proliferated in response to FLK supernatant but were no longer stimulated by p24 or by any of the peptides tested. A possible explanation would rely on intermittent episodes of \textit{in vivo} virus expression as reported for BLV-infected sheep (Powers \textit{et al.}, 1991). A similar mechanism may exist in BLV-seropositive cattle so that episodic virus expression could occur \textit{in vivo} and would be reflected by fluctuating numbers of antigen-specific T lymphocytes in the peripheral blood. In the same way, it would be conceivable that these animals may periodically harbour more T cells specific for other viral antigens (like gp51, gp30 or regulatory proteins) than for p24.

In contrast, cells from cow B73 proliferated in response to peptides 30 (146–160) and 31 (151–165) so that the stimulating T cell epitope is probably located within the sequence 146 to 165: EFVNRLQISLADNLPGVPK. Similarly, cells from cow B80 stimulated with peptides 7 (31–45) and 8 (36–50) could recognize an epitope comprised in sequence 31 to 50: PGSEQVWITLRLAILQADPT. Cells from animal B68 were activated by two different regions: one including peptides 7 (31–45), 8 (36–50), 9 (41–55) and the other represented by peptide 29 (141–155). T cell epitopes for this animal might therefore consist of sequences 31–55: PGSEQVWITLRLAILQADPTPADV and 141–155: AESYVNFNRQLISL. The antigenic specificity of the proliferative response to the different peptides was ascertained by the fact that a BLV-seronegative cow (B78, Fig. 7a) was not stimulated by any of the peptides tested.

Our results are in contrast to those reported by Gatei \textit{et al.} (1993) who observed stimulation of PBMCs by synthetic peptides derived from the gp51 envelope protein only in non-infected cattle and sheep. In BLV-seropositive sheep, they did not detect a proliferative response. These discrepancies may be explained by the fact that the authors used total PBMC and FCS in their proliferation assays. Thus a possible antigen-specific response in BLV-infected sheep might be masked by a high background proliferation with PBMCs from these animals.

The demonstration of virus-specific activated T cells in PBMCs from BLV-infected cattle with normal lymphocyte counts is in accordance with the speculation that an antigenic stimulation must regularly occur \textit{in vivo}; stimulation that is shown by the lifelong persistence of high-level anti-BLV antibodies. Other authors have suggested the implication of the cell-mediated immune response in BLV infection. Callebaut \textit{et al.} (1993) reported the existence of helper T cell epitopes on the gp51 envelope glycoprotein in BLV-infected cattle. Ohishi \textit{et al.} (1991) and Okada \textit{et al.} (1993) observed the presence of CD4\(^+\) and CD8\(^+\) T lymphocytes in delayed-type hypersensitivity skin reactions from sheep vaccinated with recombinant vaccinia virus expressing
gp51 envelope protein and challenged intradermally with BLV virions. They suggest that CD4+ T cells might be involved in the BLV growth suppression by releasing lymphokines such as interferon γ. In our study, we were able to detect activated CD4+ T cells in PBMCs and, although no information is yet available on the nature of cytokines released by these antigen-specific T cells in BLV-infected cattle with normal hematological values, this is an attractive hypothesis to account for the partial control of BLV replication during the early stages of the disease. T cell-derived lymphokines might also activate specific CTLs proposed by Ohishi et al. (1991) to be involved in the destruction of BLV-infected cells. Furthermore we speculate that the putative lymphokines could play a role in the regulation of virus expression in infected B lymphocytes. Depending on the type of cytokines released, their activity might be either to stimulate or to inhibit virus expression. Indeed, some authors argue that T cell-derived factors are necessary for the induction of BLV expression in PBMCs cultured in vitro (Cornil et al., 1988). Another hypothesis with respect to the role played by p24-specific CD4+ T cells might be their possible function as CTLs. Indeed, MHC class II-restricted CD4+ CTLs have been reported from several viral infections (Jacobson et al., 1984; Morrison et al., 1986; Littaua et al., 1992). During BLV infection, these CTLs would kill virus-producing cells (particularly infected B lymphocytes expressing MHC class II antigens), contributing to the prevention of virus spreading throughout the lymphoid system and postponing the progression toward PL.

The existence of virus-specific T cells in BLV-infected cattle with normal lymphocyte counts is another example of the important implication of cell-mediated immunity in retroviral diseases. This has already been shown for other viruses belonging to this group (B. D. Walker et al., 1987; McGraw et al., 1990; Kannagi et al., 1992). To achieve an efficient protection of cattle (and sheep) from BLV infection, it is necessary to understand the underlying natural processes of the host’s immune response. Here we reported that the p24 core protein constitutes a target antigen for CD4+ T lymphocytes, an observation worthy to be considered for vaccine development. Although no universal T cell epitope was identified, the synthetic peptide approach revealed two main regions of the p24 able to induce proliferation of T cells from the animals tested: one was located in the region of amino acids 31 to 55 (recognized by cells from animals B68 and B80) and the other at amino acids 141 to 165 (recognized by cells from animals B68 and B73). Until now, we could use only a limited number of experimental cows and their MHC class II haplotype could not be determined so that definitive conclusions cannot yet be drawn. It would be interesting to extend the identification of BLV T cell epitopes to a greater number of MHC haplotype-defined animals (cattle and sheep) representing different stages of infection in order to look for an eventual relationship between MHC haplotype, T cell epitopes, and the evolution of the disease. The resulting data would also be instructive in the design of a synthetic peptide vaccine.

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