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Cytotoxic Activity against Maedi-Visna Virus-Infected Macrophages

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The cell type predominantly infected by maedi-visna virus (MVV) is the macrophage, and we have looked at the ability of MVV-infected macrophages to interact with cytotoxic T lymphocytes (CTL), important effector cells against virus infections. MVV-specific CTL precursors were detected, after in vitro culture with MVV antigen and recombinant human interleukin-2, in peripheral blood lymphocytes of all MVV-infected sheep. MVV-infected monocyte-derived macrophages and alveolar macrophages were able to stimulate CTL activity in vitro and were targets for these activated CTL. The major effector cell population using MVV-infected macrophage targets was $CD8^+$ lymphocytes, although another population, lymphokine-activated killer cells, may also have been involved. There was no direct cytotoxic activity found in alveolar lymphocytes from MVV-infected sheep without lung lesions.

After viral infection, the host develops a humoral and cell-mediated immune response. The development of specific antibodies or cytotoxic T lymphocytes (CTL) is specifically programmed to eliminate free virus and virus-infected cells (32). Lentivirus infections are unusual in that the virus can persist in target cells, even though the host has developed virus-specific CTL and antibody (22).

Human immunodeficiency virus (HIV)-infected macrophages or monocyte cell lines can be lysed by major histocompatibility complex (MHC)-restricted virus-specific CTL (1, 27, 36), non-MHC-restricted natural killer cells, or antibody-dependent cellular cytotoxicity (9). The development of these cytotoxic activities indicates that virus-infected macrophages present viral antigen to initiate the immune response and that these cells are eliminated by activated virus-specific or nonspecific cytotoxic cells. In vivo, HIV persistence in macrophages can be detected at all stages of infection (12). Macrophages harboring virus but evading host immunosurveillance are therefore important in the pathogenesis of HIV infection.

Monocytes/macrophages are the predominantly infected cells in maedi-visna virus (MVV) infection. Virus-specific and MHC-restricted CTL activity can be detected in all MVV-infected sheep by stimulating peripheral blood mononuclear cells (PBMC) with MVV-infected skin fibroblasts (3), but direct cytotoxic activity in acute or persistent infections is very difficult to detect (2, 3). This is in contrast to the direct cytotoxic activity seen in PBMC or bronchoalveolar lavage (BAL) cells from HIV-infected patients (1, 23). There is limited viral antigen expressed on infected macrophages in vivo during MVV infection (8, 10), and so there may not be sufficient antigen to activate circulating precursors in PBMC to show CTL activity in vitro. Studies with MVV-infected sheep have shown greatly increased numbers of $CD8^+$ lymphocytes and decreasing CD4/CD8 ratios in bronchoalveolar fluids of maedi-affected lungs (19) and synovial fluid of arthritic joints (11, 15) from MVV-infected sheep. Most of these lymphocytes are activated (4, 38), which may indicate that they are functional lymphocytes with either CTL activity or suppressive effects on pulmonary immunity in MVV infection. However, there is no research showing a function for alveolar lymphocytes ($CD8^+$) in maedi. It has previously been shown that skin fibroblasts are good target cells in CTL assays with MVV (3). However, in vivo the important target cell is the infected macrophage. In this study we show that MVV-infected macrophages stimulated cytotoxic precursors in vitro and that they were also lysed by activated cytotoxic T cells.

MATERIALS AND METHODS

Sheep. Adult Finnish Landrace sheep were purchased from the Moredun Research Institute, Edinburgh. Sheep had been infected with MVV strain EV1 (31) for 0.5 to 5 years (persistently infected) as stated below.

Skin cell lines and virus culture. Autologous skin cell lines for each sheep were derived from skin biopsies and grown in Dulbecco's modified Eagle's medium (DME) with 2 mM L-glutamine, 100 U of benzyl penicillin per ml, 100 mg of streptomycin per ml, and 10% fetal calf serum (FCS) as previously described (2). Skin cell monolayers (90% confluent) were infected with MVV strain EV1 (31) at a low multiplicity of infection (2) in DME with 2% FCS. When extensive cytopathic effects were seen, the supernatant was harvested, clarified, and stored at −70°C.

Monocyte/macrophage culture. PBMC were obtained from heparinized venous blood from MVV-infected sheep by taking buffy coat cells and purifying the PBMC over Ficoll (Lymphoprep; Nycomed AS) as previously described (3). Cells were diluted in RPMI 1640 containing 2 mM L-glutamine, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 U of benzyl penicillin per ml, 100 mg of streptomycin per ml, and 5×10^{-5} M 2-mercaptoethanol (RPMI) with 10% FCS and 10% lamb serum (RPMI–10% FCS–10% lamb serum) and cultured in gelatin-coated 75-cm^2 flasks (for target cells) or 24-well plates (for stimulator cells). The gelatin coating was prepared as described by Jones et al. (13) by incubating the flasks or plates with 2% gelatin for 1 h at 37°C, removing excess fluid, drying the flasks or plates, and then before use incubating the flasks with autologous plasma for 30 min at 37°C. After 1 h of incubation at 37°C, nonadherent cells were collected as peripheral blood lymphocytes (PBL), which were used to generate MVV-specific CTL as described below.

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The adherent cells were further cultured in RPMI–10% FCS–10% lamb serum as monocyte-derived macrophages (MDM) with the medium being changed every 5 days. After 10 days of culture, MDM were treated with 5 mM EDTA in phosphate-buffered saline (PBS) for 5 min. The dislodged cells were washed once and then resuspended in RPMI–10% FCS for use as target cells.

Lymphocyte isolation and culture. PBL obtained as described above were used to generate CTL as previously described (3). Briefly, PBL were cultured with MVV-infected autologous skin cells in RPMI–10% FCS with 5 U of recombinant human interleukin-2 (rHu IL-2; NIBSC, Potters Bar, United Kingdom) per ml for 14 days, the culture being fed every 3 to 4 days. Lymphocytes were harvested, overlaid onto Lymphoprep, and centrifuged (750 × *g*, 10 min). Viable cells were harvested at the interface and washed twice with warm RPMI–2% FCS, counted in 1% trypan blue, and adjusted to the required concentrations in RPMI–10% FCS for use as effectors in CTL assays.

Alveolar lymphocytes were collected by BAL with 2 liters of cold Hanks buffered sterile saline. The cells were pelleted by centrifugation at 700 × *g* and 4°C for 10 to 20 min. Cell pellets were washed with RPMI–2% FCS three times. Alveolar lymphocytes were purified by depletion of adherent alveolar macrophages (AM) in tissue culture flasks (2×10^7 BAL cells were incubated in 175-cm^2 flasks for 1 h at 37°C). After two rounds of adherence, nonadherent cells included around 85 to 90% lymphocytes and were used as effectors in assays. The adherent cells (AM) were cultured as described for MDM, but in RPMI–20% FCS, and were used as targets in cytotoxicity assays.

The abilities of AM and MDM to activate CTL in vitro were assessed in a manner similar to that for skin cells (3) by using macrophages to present live viral antigen to autologous PBL from MVV-infected sheep. AM or MDM (cultured for 7 to 14 days) in 24-well plates were infected with MVV strain EV1 at 1 50% tissue culture infective dose per cell, for 2 h at 37°C, and then 2×10^6 PBL in RPMI–10% FCS with 5 U of rHu IL-2 per ml were added and the resulting mixture was incubated for 7 days at 37°C in 5% CO_2. Viable lymphocytes were transferred to cultures of newly infected AM or MDM and then cultured for a further 7 days as before.

Cytotoxic T lymphocyte assay. MDM, AM, and skin fibroblasts, either autologous or heterologous, were used as target cells (3). Skin fibroblasts (10^4 per well) in DME–10% FCS or MDM (1×10^4 to 2×10^4 per well) in complete RPMI–10% FCS were plated into 96-well flat-bottom microtiter plates and incubated overnight at 37°C in 5% CO_2. Cells were either mock infected or infected with MVV strain EV1 at 0.5 (skin fibroblasts) or 1 to 2 (MDM) 50% tissue culture infective doses per cell and were incubated for 24 h (skin fibroblasts) or 48 h (MDM). Each well was labelled with 1.5 μCi of $Na_2{}^{51}CrO_4$ in 50 μl of medium (DME–2% FCS and RPMI–10% FCS, respectively) and incubated overnight at 37°C in 5% CO_2. The cultures were washed four times with RPMI–2% FCS and then used as target cells in 16-h assays. Under these conditions 80% of the skin fibroblasts (3 days postinfection) and 30% of the MDM (4 days postinfection) expressed $p15^{gag}$ antigen in the cytoplasm (detected by flow cytometry). For AM targets, AM were cultured in 25-cm^2 flasks and then mock infected or infected with MVV strain EV1 at 1 50% tissue culture infective dose per cell for 3 days. AM in flasks were labelled overnight with 100 μCi of $Na_2{}^{51}CrO_4$ in 2 ml of medium at 37°C in 5% CO_2. AM were harvested, washed four times with RPMI–2% FCS, and counted, and then 2×10^4 of these AM per well were used as target cells in RPMI–10% FCS.

Effector cells were adjusted to the required cell concentrations in RPMI–10% FCS, usually by performing three dilutions, to produce effector-to-target ratios of 12:1, 25:1, and 50:1 and were added to target cells in a final volume of 200 μl (3). Plates were centrifuged (200 × *g*, 1 min) and incubated at 37°C in 5% CO_2. After 6 or 16 h of incubation, 40 μl of the supernatant was mixed with 120 μl of Optiphase Hisafe 3 (Wallac) in a 96-well plate (Falcon catalog no. 3912) and counted by scintillation counting (1450 Microbeta counter; Wallac) for 1 min. Assays were performed in triplicate for each dilution of effector cells. The minimum release was taken from wells without added effectors, and maximum release was measured by addition of 1% Triton X-100 to the targets. Results are expressed as the percent specific ^{51}Cr release, calculated by the following formula: percent specific ^{51}Cr release = [(mean cpm test − mean cpm minimum)/(mean cpm maximum − mean cpm minimum)] × 100 ± [standard deviation of cpm test/(mean cpm maximum − mean cpm minimum)] × 100.

Depletion of lymphocytes with monoclonal antibody plus complement. All reactions and washes were carried out in RPMI–2% FCS as previously described (3). Lymphocytes (1×10^7) were incubated for 40 min at 0°C in 500 μl of the following monoclonal antibodies: SBU-T4, SBU-T8 (20), or irrelevant immunoglobulin G2A (IgG2A) mouse myeloma protein (UPC10; Sigma Chemical Co.) (1 μg/ml). The cells were washed twice and then suspended in 500 μl of newborn rabbit serum (1:5 dilution) as a source of complement for 30 min at 37°C. After one wash, the complement depletion was repeated once more. The cells were washed twice. Viable cells were counted in 1% trypan blue, diluted to the required cell concentrations in RPMI–10% FCS, and used in CTL assays.

Fluorescence-activated cell sorter (FACS) analysis of cell populations. Reactions and washes were carried out in 25 μl of PBS with 0.1% bovine serum albumin and 0.01% sodium azide at 0°C. Lymphocytes were stained as described by Blacklaws et al. (3) or with monoclonal antibody SBU-T4 or SBU-T8 as described above, washed three times, and then developed with anti-mouse IgG $F(ab')_2$-fluorescein isothiocyanate conjugate (1:50 dilution; Dakopatts Ltd) for 20 min. Normal mouse serum (1:500) as the first antibody layer was used as the negative control. After three washes, cells were collected and analyzed on a Becton Dickinson FACScan. Data (10,000 cells per sample) were collected with Consort 30 version F software and analyzed with Lysys version 1.62 software. To exclude dead cells, cells were resuspended in propidium iodide (20 μg/ml) and data (7,000 cells per sample) were collected by using FACScan Research software version 2.1 excluding cells with high propidium iodide intake (3).

Activation of lymphokine-activated killer (LAK) cells. PBL were cultured in 25-cm^2 flasks at a concentration of 2×10^6 cells per ml in RPMI–10% FCS with 10 U of rHu IL-2 per ml for 5 or 13 days. More rHu IL-2 (10 U/ml) was added to the cultures every 3 to 4 days. The medium was changed once on day 7. The effector cells were overlaid onto Lymphoprep, and viable cells were harvested from the interface as effector cells. After two washes with RPMI–2% FCS, the viable cells were counted and diluted to the required concentrations for effectors in RPMI–10% FCS.

RESULTS

In vitro-activated MVV-specific CTL recognize infected macrophages. After stimulation of PBL from MVV-infected sheep (sheep no. 1091P, 865A, 1071P, and YT40) with live virus and IL-2 in vitro, the majority of the lymphocytes were

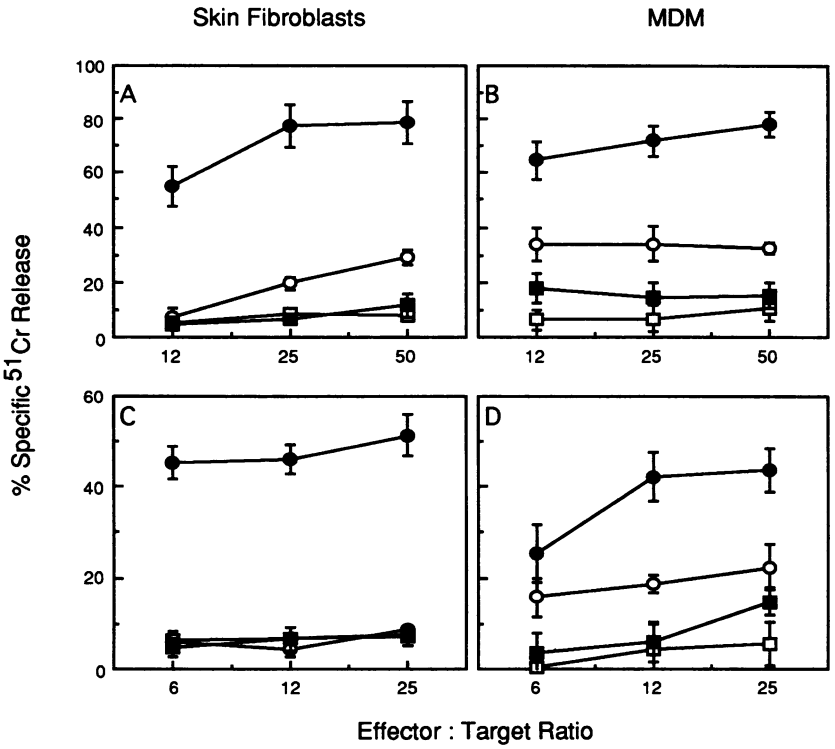

FIG. 1. Recognition of MDM targets by CTL. PBL from MVV-infected sheep (sheep 1091P [A and B] and sheep 865A [C and D]) were cultured on MVV-infected autologous skin fibroblasts as described in Materials and Methods. Target cells, autologous and heterologous skin fibroblasts (A and C) and MDM (B and D), in 96-well flat-bottom microtiter plates were prepared as described in Materials and Methods and then incubated with activated lymphocytes for 16 h. The percent spontaneous ^{51}Cr release from targets compared with maximal release was as follows: skin fibroblasts, 18 to 23%; and MDM, 23 to 32%. The results were calculated as shown in Materials and Methods. Open symbols represent uninfected targets, and closed symbols represent infected targets; results for autologous (○, ●) and heterologous (□, ■) cells are shown.

$CD8^+$ (the proportion ranged between 62 and 78%). These cell populations from all four infected sheep contained activated CTL (data for two sheep are shown in Fig. 1). They all showed virus-specific activity for autologous MVV-infected skin fibroblasts (79, 51, 64, and 46% specific ^{51}Cr release, respectively, at effector/target ratios of 50:1) but not for heterologous MVV-infected skin fibroblasts (12, 7, 8, and 15%, respectively; Fig. 1A and C). Similar results were also obtained by using MVV-infected MDM (78, 44, 63, and 42% specific ^{51}Cr release, respectively, from autologous MDM versus 15, 15, 26, and 15% specific ^{51}Cr release, respectively, from heterologous MDM; Fig. 1B and D). This indicates that the cytotoxic activity is MHC restricted on both fibroblasts and macrophages. However, levels of nonspecific cytotoxic activity on uninfected macrophages were often high (10 to 35%), especially on autologous macrophages, although this nonspecific activity varied in different sheep (Fig. 1B and D). Sometimes, at high effector-to-target ratios, nonspecific cytotoxicity was also seen on autologous uninfected skin fibroblasts rather than on heterologous skin fibroblasts (Fig. 1A).

In the above-described assays, autologous MDM were derived from MVV-infected sheep. These macrophages may therefore have expressed low levels of MVV antigen without infection in vitro because of persistent MVV infection of the monocytes in vivo. Indeed, when MDM from MVV-infected sheep were stained for *gag* antigen (p15), occasional cells were *gag* positive (less than 1%; data not shown). Therefore, some of the background nonspecific killing of autologous MDM described above may in fact have been virus specific. To test this hypothesis, MDM were derived from an uninfected sheep whose skin fibroblasts were known to present antigen to the effector lymphocytes (1238T MDM with 1071P CTL; data not shown). The results showed similar levels of nonspecific lysis with uninfected autologous (1071P) and cross-reacting (1238T) MDM targets (data not shown). Therefore, the nonspecific cytotoxicity seen with autologous MDM targets was not caused by virus infection of monocytes in vivo.

Although recognition of MDM had been shown to occur, in vivo the natural target for CTL, especially in sheep with maedi, might be expected to be AM. Therefore, AM were used as targets for in vitro-stimulated CTL from blood. MVV-infected autologous AM were shown to be specifically lysed by CTL when they were compared with autologous uninfected AM and infected heterologous AM (Fig. 2). Spontaneous ^{51}Cr release from AM was often high (data not shown), and so assay incubations were reduced to 6 h.

Generation of MVV-specific CTL by using autologous macrophages. To address whether MVV-infected macrophages could stimulate virus-specific precursor CTL in vitro, autologous MDM (Fig. 3A) and AM (Fig. 3B) infected with MVV were used as stimulating cells to activate precursor CTL from PBMC. After 2 weeks of stimulation, the $CD8^+$ lymphocyte populations in the cultures were increased to 65% (sheep YT40) and 66% (sheep 1257J), respectively. An apparently MHC-restricted CTL activity was detected in effectors cultured on MDM by using virus-infected skin fibroblast targets (YT40; data not shown) or MDM targets (Fig. 3A), and virus-specific lysis was seen with effectors cultured on AM (Fig. 3B). Levels

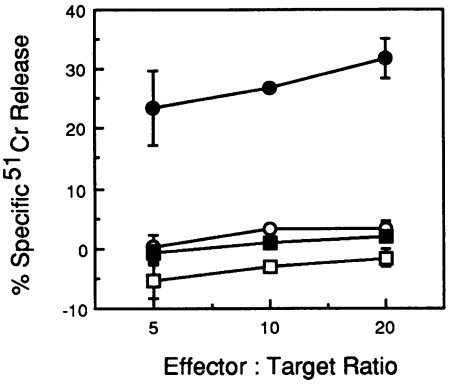

FIG. 2. Recognition of AM targets by CTL. Effector cells (sheep 865A) had been stimulated on MVV-infected autologous skin fibroblasts as described in Materials and Methods. Autologous (865A) and heterologous (1250J) AM were resuscitated after storage in liquid nitrogen, cultured in 25-cm^2 flasks, and then infected and used as target cells as previously described. Effector cells were added and incubated with target cells for 6 h. The spontaneous ^{51}Cr release was 21 to 24% compared with the maximal release. The results were calculated as shown in Materials and Methods. Open symbols represent uninfected targets, and closed symbols represent infected targets; results for autologous (○, ●) and heterologous (□, ■) cells are shown.

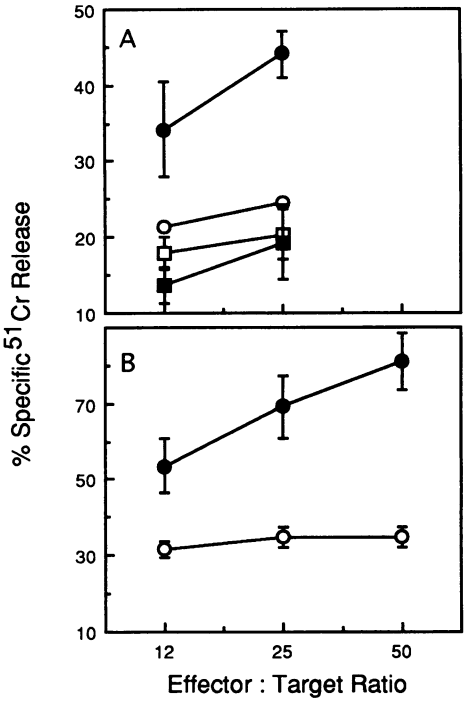

FIG. 3. Stimulation of precursor CTL by macrophages in vitro. PBL were stimulated on MVV-infected autologous MDM (sheep YT40 [A]) or AM (sheep 1257J [B]) in the presence of IL-2 for 2 weeks as described in Materials and Methods. Target cells, autologous and heterologous MDM, were prepared as previously described. Effector and target cells were incubated together for 16 h. The percent spontaneous ^{51}Cr release from targets compared with the maximal release ranged from 25 to 32%. The results were calculated as shown in Materials and Methods. Open symbols represent uninfected targets, and closed symbols represent infected targets; results for autologous (○, ●) and heterologous (□, ■) cells are shown.

of nonspecific lysis of autologous uninfected MDM could be high (around 15 to 30%), as was also seen with lymphocyte effectors cultured on skin fibroblasts (Fig. 1 and 3).

CD8$^+$ lymphocytes mediate CTL activity. To investigate the phenotype of the CTL, CD4$^+$ or CD8$^+$ lymphocytes were depleted from cultured effector cell populations by complement. The results (sheep 1071P) are shown in Tables 1 and 2. When skin-cell-activated CTL were used, depletion of CD8$^+$ effector cells reduced the lysis of MVV-infected skin cell targets to background levels; however, there was apparently still some CTL activity on MVV-infected MDM targets, which would suggest that CTL activity was caused mainly by CD8$^+$ lymphocytes but that there was a contribution by another cell type. This minor cell population could be CD4$^+$ lymphocytes, as there was a slight reduction in the percent specific ^{51}Cr release at the lower effector/target ratio with CD4$^+$-depleted effector cells on MVV-infected MDM targets.

Skin fibroblasts express little, if any, MHC class II, and therefore the CD8$^+$ CTL phenotype described above is not surprising (3). However, macrophages are able to present viral antigens via both MHC class I and MHC class II and so should be able to stimulate both CD4$^+$ and CD8$^+$ virus-specific precursor CTL in vitro. Effector cell populations activated on MDM were therefore studied, and this examination showed that CD8$^+$ lymphocytes mediated MVV-specific cytotoxic activity (Table 1, results for effectors depleted of CD8$^+$ cells are in the SBU-T8 column, and Table 2). There was little evidence of cytotoxicity mediated by CD4$^+$ lymphocytes with these effector populations.

Cytotoxic activity of alveolar lymphocytes from MVV-infected sheep. Only a low frequency of MVV-infected sheep show direct CTL activity by uncultured PBMC (3). To investigate whether alveolar lymphocytes could mediate CTL activity without in vitro stimulation, three sheep (111, YT40, and 865A) which had been infected with MVV for more than 4 years were used to obtain BAL fluid. None of these sheep showed clinical symptoms, and the lungs had no gross lesions. Alveolar lymphocytes were collected, purified by removing adherent cells, and then used as effector cells. Skin fibroblasts and MDM, either autologous or heterologous, were used as target cells, but no virus-specific MHC-restricted cytotoxic activity was detected with most sheep (Fig. 4). Low levels of CTL activity were seen with sheep YT40 when skin fibroblast targets were used (Fig. 4B), but the difference in lysis between uninfected and infected targets did not exceed 10% specific ^{51}Cr release.

LAK cells mediate cytotoxicity. To study whether the nonspecific cytotoxic activity seen with autologous and heterologous uninfected MDM was mediated by LAK cells, PBL were cultured with rHu IL-2 (10 U/ml) for 5 to 13 days and then used as effector cells (LAK cells). These results showed various levels of nonspecific lysis of MVV-infected or mock-infected targets (autologous and heterologous) in different sheep. Autologous MVV-infected MDM were more susceptible than other targets in the case of sheep 647R (Fig. 5A). Sheep 647R was MVV infected, and it is known to have precursor CTL in its blood, which may have caused this result. Sheep 1138W was uninfected, and precursor CTL have never been detected in the blood of uninfected sheep (3). All MDM targets were lysed to the same extent by LAK cells from sheep 1138W (approximately 40% specific ^{51}Cr release at effector/target ratios of 50:1; Fig. 5B and C). Skin fibroblasts, especially MVV-infected cells, were also lysed by LAK cells, but the levels of killing were relatively low compared with those of MDM targets (Fig. 5C). However, the susceptibility of macrophages to lysis by LAK cells was maturation dependent; immature MDM (cultured for

TABLE 1. Percent specific ^{51}Cr release by lymphocyte subpopulation-depleted effectors

Autologous target and type of infection	Effector/target ratio	% Specific ^{51}Cr release by CTL incubated with the following antibody (mean ± standard deviation)[a]:					
		Skin fibroblast-activated CTL			MDM-activated CTL		
		IgG2A	SBU-T4	SBU-T8	IgG2A	SBU-T4	SBU-T8
MDM							
Mock infected	8:1	13.2 ± 3.5	17.3 ± 1.7	12.0 ± 8.3	14.6 ± 5.8	13.8 ± 4.3	15.7 ± 3.9
	16:1	16.4 ± 4.7	23.8 ± 5.1	14.1 ± 12.8	19.8 ± 2.1	17.9 ± 2.3	14.7 ± 3.5
MVV infected	8:1	44.2 ± 3.7	37.0 ± 5.1	14.9 ± 3.3	52.6 ± 4.8	46.1 ± 3.5	14.7 ± 2.6
	16:1	44.5 ± 4.9	47.4 ± 4.0	24.2 ± 2.8	66.6 ± 2.3	52.0 ± 8.3	19.6 ± 5.2
Skin fibroblasts							
Mock infected	8:1	18.0 ± 3.8	20.6 ± 4.6	2.0 ± 0.9	5.9 ± 0.3	6.9 ± 0.5	4.7 ± 1.0
	16:1	24.4 ± 1.1	21.7 ± 1.9	4.6 ± 0.8	8.5 ± 1.9	5.5 ± 1.2	5.0 ± 0.8
MVV infected	8:1	45.6 ± 4.6	49.1 ± 3.7	4.2 ± 2.5	41.2 ± 4.0	43.6 ± 3.1	11.8 ± 3.1
	16:1	48.7 ± 4.9	54.1 ± 0.3	5.6 ± 0.9	50.8 ± 2.8	42.9 ± 1.8	11.2 ± 0.3

[a] PBL were stimulated on autologous MVV-infected skin fibroblasts or MDM in the presence of rHu IL-2 for 2 weeks as described in Materials and Methods. Activated CTL were incubated with monoclonal antibody, irrelevant mouse IgG2A, SBU-T4 (anti-CD4), or SBU-T8 (anti-CD8) followed by rabbit serum as a source of complement before use in assays. Spontaneous ^{51}Cr release was between 20.2 and 25.7%.

5 days) were more resistant to lysis than mature MDM (cultured for 12 to 14 days) (data not shown). Therefore, LAK cells do contribute to the high background levels of lysis seen with macrophage targets.

DISCUSSION

In this report we describe the interaction of macrophages and cytotoxic T cells during lentivirus infection. Under certain conditions macrophages can act to inhibit immune responses, and macrophage activities may be involved in some aspects of primary immunodeficiency (28). MVV is a persistent virus infection in which the virus is never completely cleared from its infected cell type, the macrophage. This persistence may be due to several interacting mechanisms, for example, latent or restricted virus replication, virus escape mutants, or deficient immune responses to the virus. Little is known about the T-cell, and especially the CTL, response to MVV, and so we have looked at the ability of macrophages to stimulate CTL activity in vitro (Fig. 3). Precursor CTL from MVV-infected sheep were stimulated consistently by autologous MVV-infected AM and MDM. Therefore, the in vivo-infected cell lineage can stimulate precursor CTL to become active CTL. Previous studies have used skin fibroblasts (3, 17) or MDM (14) to present antigen during in vitro stimulation of precursor CTL.

Similarly, both AM and MDM acted as targets for MHC-restricted lysis by MVV-specific CTL (Fig. 1 and 2). AM have been used successfully as targets with HIV-specific CTL (1, 27). AM do not present antigen well to T cells in proliferation assays (via MHC class II), but here, when infected, they efficiently presented endogenously produced viral antigen to CTL. By using skin fibroblasts, the MVV-specific CTL population has been shown to be $CD8^+$ (3). Skin fibroblasts do not express MHC class II molecules, as determined by FACS analysis, and therefore the use of the MHC class I-$CD8^+$ interaction is not surprising. However, AM and MDM express MHC class II especially when cultured with lymphocytes, and so they were used to assess the role of $CD4^+$ CTL during MVV infection. We could not prove that any cytotoxicity was mediated by $CD4^+$ cells with MDM-activated CTL (from MVV-infected sheep). In addition, an MVV-specific T-cell line (75% $CD4^+$ T cells and 8% $CD8^+$ T cells) generated from a $p25^{gag}$-primed uninfected sheep did not show any cytotoxic activity (data not shown). These results are in contrast to those for HIV, since $CD4^+$ CTL-mediated cytotoxicity can be detected in HIV-infected and antigen-primed situations (5, 18, 21, 24).

Skin fibroblast targets produced lower backgrounds, less variability, and better viral infections than AM and MDM targets, and so results were much clearer when fibroblast targets were used. There was often lysis of uninfected autologous macrophages, which confused results. Depletion of $CD8^+$ effector cells caused a decrease in recognition of MVV-infected autologous MDM but did not reduce the level of ^{51}Cr release to background levels (Table 1). The same $CD8^+$ depletion with MVV-infected skin fibroblast targets reduced cytotoxicity to background levels. Therefore, other effectors involved in non-MHC-restricted cytotoxicity of macrophages may also have been present in the culture (for example, LAK cells).

In vitro it is often necessary to add IL-2 to cultures to generate virus-specific CTL (7). However, this can also lead, especially in the absence of virus-infected stimulator cells, to the activation of LAK cells (34). IL-2 has been reported to induce functionally active LAK cells against tumor cells or virus-infected cells (30, 35). LAK cells also show nonspecific cytotoxic activity against macrophages, especially autologous macrophages, whether they are infected with virus or not (6, 33).

The frequency of LAK cells in PBL cultures stimulated by MVV-infected skin fibroblasts in the presence of IL-2 may be

TABLE 2. Phenotype of effector cell populations[a]

Type of CTL	Antibody used for depletion	% of effector cell population with the following phenotype[b]:	
		$CD4^+$	$CD8^+$
Skin fibroblast activated	IgG2A	25.0	70.5
	SBU-T4	0.2	92.6
	SBU-T8	71.1	0.2
MDM activated	IgG2A	14.7	85.0
	SBU-T4	0.0	97.4
	SBU-T8	80.4	9.1

[a] PBL were stimulated and then depleted as described in footnote *a* to Table 1.

[b] Effector cell phenotype was analyzed by flow cytometry.

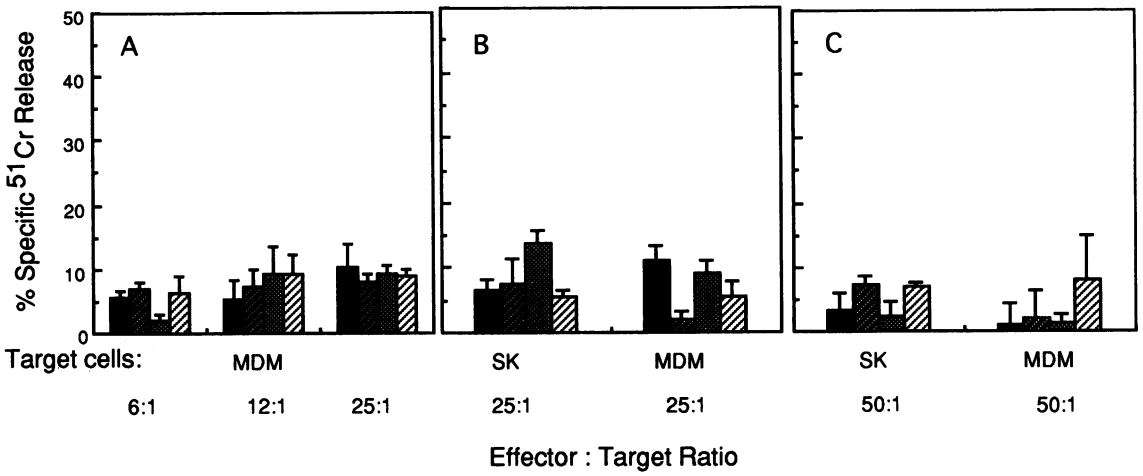

FIG. 4. Alveolar lymphocyte effectors from MVV-infected sheep. Alveolar lymphocytes were purified from BAL fluid from MVV-infected sheep (sheep 111 [A], YT40 [B], and 865A [C]) by adherence of AM to plastic plates or tissue culture flasks twice, and then nonadherent cells were used as effector cells. Target cells, autologous and heterologous skin fibroblasts (SK) or MDM (as indicated), were infected or mock infected with MVV as described in Materials and Methods. Assays were incubated for 16 h. The percent spontaneous ^{51}Cr release from targets compared with the maximal release was as follows: MDM, 22 to 31%; skin fibroblasts, 20 to 23%. The results were calculated as shown in Materials and Methods. Symbols: ■, autologous control; ▨, heterologous control; ▩, autologous, EV1 infected; ▨, heterologous, EV1 infected.

different from that of LAK cells in cultures stimulated with only IL-2, as lymphocytes stimulated by the viral antigen may themselves be producing interleukins. Hence, it is difficult to evaluate the contribution of LAK cells to the levels of nonspecific activity involved in the CTL assays described here. An antibody-dependent cellular cytotoxicity assay (26) with HIV using U937 (a monocytic cell line) as target cells showed the occurrence of around 35% nonspecific lysis with uninfected cells (9), but the reason for this lysis has not been discussed. High nonspecific backgrounds have also been reported with macrophages lysed by IL-2-activated effector cells (16) and with autologous monocyte targets during a $CD4^+$ CTL-mediated cytotoxicity assay (25). These reports support the view that the high levels of background lysis obtained here with MDM targets were within normal ranges.

Two distinct effectors mediating simian immunodeficiency virus (SIV) *env*-specific target cell lysis have been reported (40). One effector, which is similar to natural killer cells, is $CD16^+$ and MHC class I unrestricted. Weinhold et al. (39) showed that $CD4^+$ lymphocytes coated with HIV gp120 were lysed by a population of non-T-cell effectors. This cytotoxicity was enhanced after activation of effector cells with IL-2, but it greatly decreased after the depletion of $CD16^+$ cells. In contrast, Riviere et al. (29), using a monoclonal antibody for CD3 which was incubated with effector cells to block reactions, inhibited *gag*-specific cytotoxic activity but not *env*-specific cytotoxicity. The depletion of $CD8^+$ cells had no effect on cytotoxic activity against autologous target cells infected with vaccinia virus expressing *env* protein. However, there are also reports of MHC-restricted CTL specific for HIV *env* (23). These results suggest that two distinct types of effector cells mediate the killing of targets expressing *gag* and *env* proteins. Natural killer cells or LAK cells play a significant role in controlling *env* protein-expressing cells, while classical CTL kill both *gag* protein-expressing cells and *env* protein-expressing cells. Here, the levels of cell lysis mediated by LAK cells from MVV-infected sheep seen with virus-infected autologous MDM (Fig. 5) were higher than those seen with mock-infected controls, and this result may be due to an *env*-specific effect as seen with HIV and SIV, although the occurrence of some CTL activity in these effector cell populations cannot be ruled out.

CTL activity in lymphocytes not subjected to in vitro stimulation is rare in PBMC from MVV-infected sheep (3) and also in lymphocytes draining from acutely infected lymph nodes (2). Additional stimulation of precursor cells in vitro is necessary to produce CTL activity in most MVV-infected sheep. This was also found to be the case with caprine arthritis-encephalitis virus, the lentivirus most closely related to MVV (17). In HIV, 15 to 85% of patients may show killing without secondary in vitro stimulation (23), and CTL activity without in vitro stimulation has also been reported in PBMC with SIV (37). Both HIV and SIV replicate in PBMC, and it was therefore thought that if lymphocytes from a major site of MVV replication were studied, sufficient antigen might be presented in vivo at such sites to stimulate CTL activity, which could then be seen directly in vitro in a larger proportion of cases. None of the three sheep analyzed showed clear CTL activity in alveolar lymphocytes (Fig. 4). However, none of the lungs obtained showed gross maedi lesions (interstitial pneumonia), and therefore they may not have been the best samples to use: clinical scoring of sheep for maedi is difficult and inaccurate, and advanced maedi cases usually do not develop because of management and control of experimental sheep. There was no significant increase in the numbers of $CD8^+$ lymphocytes in the BAL fluid of sheep without lung lesions, and the numbers of lymphocytes recovered from these lungs were also low and so effector-to-target ratios were low. When unstimulated PBMC are used, high effector-to-target ratios (100:1) are needed to produce specific lysis of MVV-infected cells (3). Here, these levels were never reached, as lymphocytes are a minor population in lung fluids. The lack of CTL activity here may also have been partially due to this.

In contrast, active CTL have been reported to be present in alveolar lymphocytes without secondary in vitro stimulation in HIV-infected patients with interstitial pneumonia at low effector-to-target ratios (27). HIV-infected patients with interstitial pneumonia display an abnormally high level of infiltration of $CD8^+$ $D44^+$ T lymphocytes, which suggests that these cells are

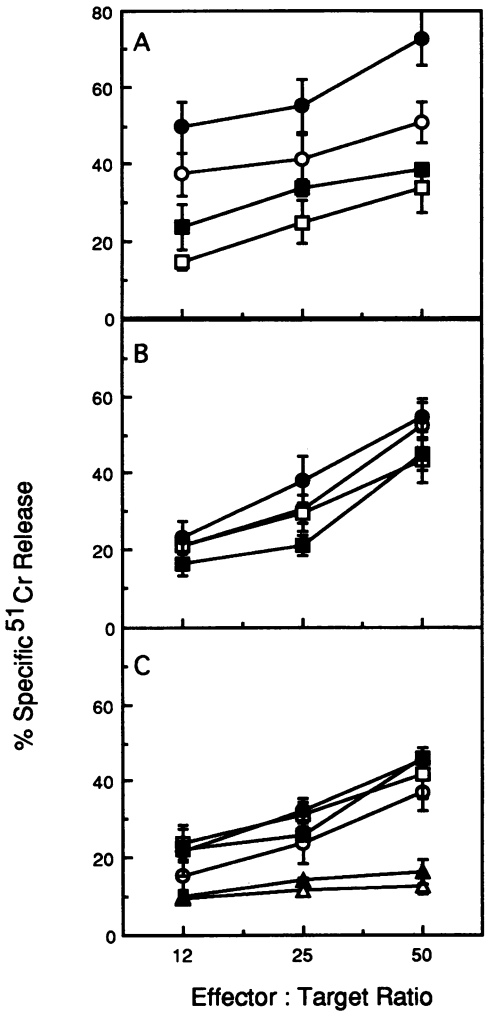

FIG. 5. Cytotoxic activity mediated by LAK cells. LAK cells were stimulated in the presence of rHu IL-2 (10 U/ml) for 5 days (sheep 647R [A] and 1138W [B]) or 13 days (sheep 1138W [C]), respectively, as described in Materials and Methods. Target cells, autologous and heterologous MDM (A through C) and autologous skin fibroblasts (C), were prepared as described in Materials and Methods. Assay mixtures were incubated for 16 h. The percents spontaneous ^{51}Cr release from targets compared with the maximal release were as follows: 27 to 31% (A), 26 to 30% (B), and 18 to 25% (C). The results were calculated as shown in Materials and Methods. Open symbols represent uninfected targets, and closed symbols represent infected targets; results for autologous (○, ●) and heterologous (□, ■) MDM and autologous skin fibroblasts (△, ▲) are shown.

a subset of CTL. In a manner similar to that of the results shown here, however, CTL activity mediated by alveolar lymphocytes cannot be detected in HIV-seropositive patients without interstitial lung lesions (1). Hence, the results shown here cannot be used to indicate whether there will be a lack of CTL activity in the lungs of MVV-infected sheep with pulmonary lesions. Patients with CTL activity in their alveolar lymphocytes also showed high levels of AM expressing viral antigen (12 to 58%) (27). It is these viral antigen-expressing AM which may be stimulating alveolar lymphocytes to become active CTL. However, a high frequency of AM expressing viral antigen is not seen in MVV-infected sheep with lung lesions (38), and this observation would suggest that CTL detected without in vitro stimulation may be rare even in the case of alveolar lymphocytes from lungs with maedi.

We have shown that macrophages, both MDM and AM, can stimulate precursor CTL to become activated effectors and can be lysed by activated CTL. Therefore the in vivo-infected cell lineage of MVV may be cleared by a CTL mechanism, and this could be a reason why productively infected macrophages are rarely seen in vivo in MVV infections.

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