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Initial Lentivirus-Host Interactions within Lymph Nodes: a Study of Maedi-Visna Virus Infection in Sheep

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Reactive changes occurring within lymph nodes draining the subcutaneous site of acute infection with maedi-visna virus (MVV) were studied, and the appearance of infected cells correlated with the immune response. Cells infected with virus were detected in the node by cocultivation from day 4 postinfection (p.i.), with maximum numbers being seen between days 7 and 14, but even then infected cells were rare, with a maximum frequency of 23 50% tissue culture infective doses ($TCID_{50}$) in 10^6 lymph node cells. At later times, infected cells were still detected, but their numbers fell to 1 to 2 $TCID_{50}$ per 10^6 cells. Virus-specific $CD8^+$ cytotoxic T-cell precursors (CTLp) were isolated from infected nodes from day 10 p.i. onwards, and T-cell proliferative responses to MVV were first detected on day 7 and consistently detected after day 18. Histological analysis showed a vigorous immune response in the node. There was a marked blast reaction in the T-cell-rich zones, which was greatest at the time when the number of virally infected cells was at its height. At this stage, large numbers of plasma cells were seen in the medullary cords, indicating that extensive T-cell-dependent B-cell activation was occurring in the T-cell-rich zones. Germinal centers were prominent shortly after the onset of the T-zone response and were still present at 40 days p.i. Phenotype studies of isolated lymph node cells failed to detect major changes in the proportion or phenotype of macrophages, $CD1^+$ interdigitating cells, and $CD4^+$ or $CD8^+$ T cells despite the fact that $CD8^+$ lymphoblasts form a major population leaving the node in efferent lymph. This suggests that there is a balanced increase in the number of all cell types in response to the virus within the node and selective migration of $CD8^+$ lymphoblasts containing virus-specific CTLp from the node. Virus-specific immune responses are therefore present within the node when infectious virus isolation is maximal, but cellular immunity may act to control the level of infection from day 18 onwards.

The usual course of virus infections can be considered to follow a number of stages: first, infection and replication of virus; second, viral antigens (or infected cells) induce an immune response, and lastly, immune effector mechanisms clear the virus (9, 40). Although most virus infections could be said to follow this strategy, some immune responses are pathological for the host (e.g., lymphocytic choriomeningitis virus infection in adult mice) (2, 23), and some viruses evade the immune response and so are not cleared from the host (herpesviruses can become latent in the nervous system or lymphoid cells) (35). Of even more interest are those viruses, such as lentiviruses (29), which evade host immune responses and persist even though sites of true latency have not been found. In these cases, the virus continues to replicate, and there is a gradual buildup of pathology. The lentivirus family includes the human, simian, and feline immunodeficiency viruses (HIV, SIV, and FIV, respectively) and the small ruminant lentiviruses maedi-visna virus (MVV) and caprine arthritis encephalitis virus.

Specific immune responses are induced in lymphoid tissue, and it is also in lymphoid tissue that continuing lentiviral replication occurs during disease progression (11, 31). For humans infected with HIV, little is known about the initial stages of infection and induction of immunity because of the subclinical nature of the infection. It is important to understand the viral and immunological events that occur in vivo in lymphoid tissue during primary lentiviral infection that result in viral persistence in order to devise realistic vaccination strategies (12). We are studying the very early interaction of the lentivirus MVV with the host immune response within sheep lymphoid tissue. Sheep are amenable to surgical lymphatic cannulation, and therefore such a detailed analysis is possible. Previously we have shown that major histocompatibility complex (MHC) class II-positive $CD8^+$ lymphoblasts exit the acutely infected lymph node via efferent lymph at about the same time that low frequencies of virus-infected cells are also detected leaving the node, disseminating the infection (4). It is now appreciated that the lymph nodes are major sites of HIV replication throughout the disease (11, 31), and it is therefore likely that the virus-infected cells seen leaving acutely MVV-infected nodes are only a small proportion of the infected cells which remain in the node. We have therefore looked, first, for the establishment and maintenance of MVV infection within the lymph node and, second, for abnormalities in the immune response to MVV during acute infection of the node which might prevent viral clearance.

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MATERIALS AND METHODS

Animals. Finnish Landrace sheep (1 to 5 years of age) were purchased from the Moredun Research Institute, Edinburgh, Scotland.

Skin cell lines and virus culture. Autologous skin cell lines for each sheep were derived from skin biopsies as previously described (4). Autologous cell-grown MVV strain EV1 (36) was cultured as previously described (4) in Dul-

becco's modified Eagle's medium (DME) supplemented with 2 mM L-glutamine, 100 U of benzylpenicillin plus 100 U of streptomycin per ml, and 2% fetal calf serum (FCS) (2% DME). Virus was stored at −80°C and diluted in 2% DME to give 2.5×10^4 50% tissue culture infective doses ($TCID_{50}$)/ml for injection into sheep on the day of use. Virus was inactivated for 1 h at room temperature under a short UV wavelength bulb (Philips TUV 30W/G30 T8). No infectious virus was detectable after this treatment, and the stock was diluted for injection according to its original virus titer.

Infection of sheep and preparation of lymph nodes. Sheep were infected with MVV (4 ml of virus-infected autologous cell supernatant, total 10^5 $TCID_{50}$) at four sites within the drainage area of the prefemoral lymph node. The prefemoral lymph nodes were excised, excess fat was removed, and the nodes were weighed. Nodes were sectioned transversely, and both end pieces were put into Hanks' buffered sterile saline (HBSS). More central sections were snap frozen in liquid nitrogen and stored at −80°C or fixed in neutral buffered formalin overnight before being embedded in wax blocks and stored at room temperature. The end node sections were removed from HBSS, chopped into small pieces with sterile scissors in 1 ml of RPMI 1640 with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; catalog no. 12-604-54; ICN Flow) supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U of benzylpenicillin per ml, 100 U of streptomycin per ml, and 10% FCS (10% RPMI), and then teased apart with sterile forceps in three changes of 1 ml each of 10% RPMI. The cells removed from the node were pooled, and the resulting node lumps were digested for 30 min in 10% RPMI with 10 μg of collagenase XI (Sigma C7657) and 20 ng of DNase I (Sigma D5025) per ml at room temperature with continuous agitation. These lumps were again teased apart with forceps in three changes of 1 ml each of 10% RPMI, and the cell suspensions were pooled with the previous suspensions. Cells were purified over Lymphoprep (Nycomed) ($800 \times g$, 10 min), and interface cells were washed twice in RPMI supplemented as above but with 2% FCS (2% RPMI) before suspension in the required medium for use immediately or storage in 10% dimethyl sulfoxide–90% FCS under liquid nitrogen. Preliminary experiments showed that a 30-min digestion with collagenase gave single-cell suspensions with maximal levels of immunoglobulin production in vitro and good proliferative results and that purification of the cells on Lymphoprep did not remove significant numbers of large cells.

Quantitation of infectious MVV. Infectious virus was detected by cocultivation of serial dilutions of isolated lymph node cells on heterologous skin fibroblast monolayers in DME supplemented as above but with 3% FCS as previously described (4) followed by detection of virus by staining for MVV *gag* p15.

CTL assays. Cytotoxic T-lymphocyte (CTL) assays were performed as previously described (5). Briefly, lymphocyte effectors were made to the required concentration for effector-target cell ratios of 3:1 to 100:1 before incubation with target cells for 16 h at 37°C in 5% CO_2. Targets were 10^4 autologous or heterologous skin cells either mock infected or infected for 72 h with 0.5 $TCID_{50}$ of MVV EV1 per cell labelled overnight with 1 μCi of ^{51}Cr (sodium chromate; 229 to 550 mCi/mg of Cr; ICN Biomedicals, Inc.) per 10^4 cells. Targets were washed four times and then incubated with effectors in 10% RPMI in a total volume of 150 μl. The radioactivity in 40 μl of supernatant was counted in a Wallac 1450 Microbeta liquid scintillation counter. The results have been expressed as the percent MVV specific cytotoxicity, calculated as percent specific ^{51}Cr release from infected targets minus percent specific ^{51}Cr release from mock-infected targets ± the sum of their standard deviations. All values were obtained from triplicate samples, and spontaneous ^{51}Cr release was always less than 30% of maximum release.

Effectors were uncultured lymph node cells or lymph node cells cultured for 14 days on autologous MVV EV1-infected skin fibroblasts in 10% RPMI with 5 U of recombinant human interleukin-2 (IL-2) per ml (5).

Proliferation of lymph node cells to MVV antigen. Lymph node cells were resuspended in 10% RPMI supplemented with 200 μg gentamicin per ml, and 10^5 cells were plated per well into flat-bottomed microtiter plates. MVV or mock-infected antigen was added to a final concentration of 1.75, 3.5, or 7.0 μg of protein per ml in 200 μl, and the cells were cultured for 5 days at 37°C in 5% CO_2. Cultures were pulsed with 1 μCi of [^{3}H]thymidine (20 to 30 Ci/mmol; Amersham International PLC) per well for the final 5 h, and harvested onto glass fiber filters with a Tomtec Harvester 96 automatic cell harvester, and radioactivity was counted with a Wallac 1450 Microbeta liquid scintillation counter. Results have been expressed as the stimulation index of MVV antigen-stimulated cultures compared with mock antigen-stimulated cultures (mean counts per minute [cpm] for MVV-stimulated wells/mean cpm for mock-stimulated wells) for each antigen dilution. Results were obtained from quadruplicate wells.

MVV and mock-infected antigens were prepared from the same sheep fibroblast cell line. Supernatants of infected cell cultures (and cultures mock infected at the same time) showing extensive cytopathic effects were clarified by centrifugation at $10,000 \times g$ at 4°C for 30 min, and virus was then pelleted at $10,000 \times g$ at 4°C for 16 to 18 h, resuspended in phosphate-buffered saline (PBS), and stored at −80°C. The protein concentrations of each sample were determined by using a Bio-Rad Microprotein assay kit, and the optimum concentrations for use in a proliferation assay were determined from trial assays with lymph node cells from persistently infected sheep.

Histology. Sections of the wax-embedded tissues were cut and stained by the methyl green pyronin method, which allows lymphoblasts and plasma cells to be readily identified on the basis of their high mRNA content and decondensed chromatin. The sections were assessed by an independent observer who had no knowledge of the time of infection of the sheep. Uninfected lymph node sections were randomly included as controls.

Immunohistology was performed on 8-μm-thick frozen sections by using monoclonal antibody (MAb) 415 anti-MVV *gag* p15, kindly donated by D. J. Houwers, in combination with biotin-conjugated rabbit anti-mouse immunoglobulin (Sigma), streptavidin-horseradish peroxidase conjugate (Sigma), and 3,3′-diaminobenzidine tetrahydrochloride (Sigma) as substrates.

Immunofluorescence analysis of cell phenotype. The phenotype of cells isolated from the lymph nodes was determined by using a panel of MAbs against sheep surface molecules (Table 1). Analysis was carried out on a Becton Dickinson FACScan (Becton Dickinson, Mountain View, Calif.). Ten thousand cells, with dead cells excluded on the basis of forward scatter (FSC), and 4,000 large cells defined by a gate based on their higher FSC and side scatter (SSC) properties (see Fig. 5A) were analyzed per lymph node with Consort 30 version F (Becton Dickinson). MHC class II DR and DQ and IL-2 receptor expression were analyzed on strongly positive $CD4^+$ and $CD8^+$ lymphocytes by two-color analysis as described previously (4).

TABLE 1. Specificity of MAbs for cell phenotyping

MAb clone	Specificity	Reference
OM1	CD11c α chain	17
VPM65	CD14	16
VPM5	CD1	7
SBU-T4	CD4	27
SBU-T8	CD8	27
T197	T19	25
DU2-104	Pan B	26
VPM36	MHC class II DQ	10
VPM54	MHC class II DR	10
ILA-111	IL-2R CD25	28

RESULTS

A series of prefemoral lymph nodes were obtained at different times after local MVV infection (4 days to approximately 18 months p.i.). Each infected node was from a different sheep (two sheep at each time point removed on different days), and eight prefemoral nodes were removed from uninfected sheep as controls. In addition, two sheep were mock infected with UV-inactivated MVV prior to lymph node removal. Single-cell suspensions derived from the nodes were used to analyze the appearance of infected cells and the specific immune response to MVV, and these were correlated to histological changes within the nodes.

Time course of virus output. Cell-associated virus could be detected by cocultivation in all but two infected lymph nodes

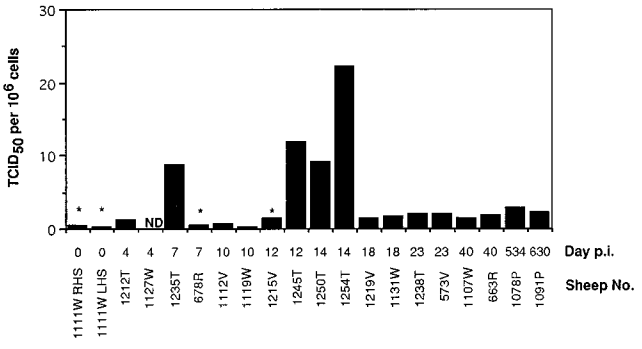

FIG. 1. Frequency of infectious MVV cells recovered from MVV-infected lymph nodes. Infectious virus was detected by cocultivation of stored lymph node cells on sheep skin fibroblast monolayers for 12 to 14 days, after which the monolayers were stained by immunofluorescence for MVV *gag*p15. *, no MVV detectable by cocultivation. The day p.i. is shown on the *x* axis, with individual sheep numbers indicated. ND, not done.

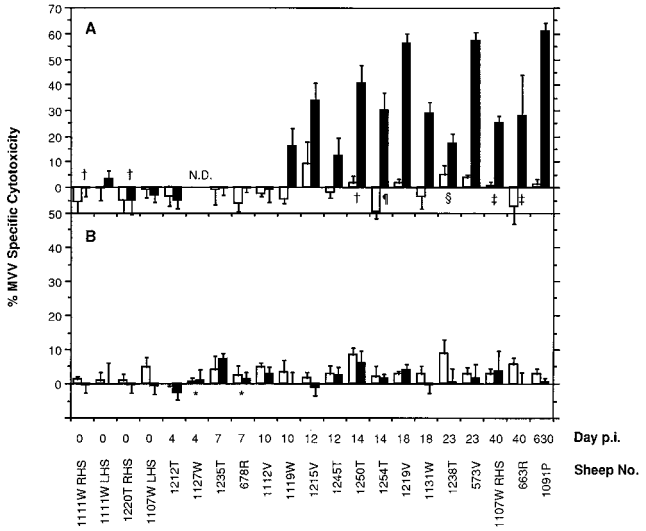

FIG. 2. Appearance of CTL in MVV-infected lymph nodes. Lymph node cells were either cultured for 14 days with live viral antigen and IL-2 (A) or used directly from the node (B) in 16-h cytotoxic assays. Targets were autologous (solid bars) or heterologous (open bars) skin cells either mock infected or infected with 0.5 $TCID_{50}$ of MVV strain EV1 per cell for 72 h at the end of the assay. Lymph node cell effectors were used at various effector-target cell ratios of 25:1 (A) and 50:1 (B) or the maximum values indicated: *, 25:1; ¶, 20:1; †, 12:1; ‡, 10:1; or §, 6:1. The day p.i. is shown on the *x* axis, with individual sheep numbers indicated. N.D., not done.

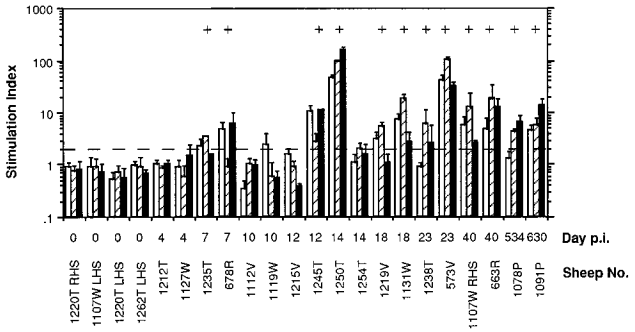

FIG. 3. Lymph node cell proliferation to MVV antigen. A total of 10^5 cells from lymph node single-cell suspensions were cultured with both MVV- and mock-infected antigen at antigen dilutions of 1.7 (open bars), 3.5 (hatched bars), and 7.0 (solid bars) μg of protein per ml for 5 days before being labelled for 5 h with [^{3}H]thymidine and harvested. Results are expressed as the stimulation index (MVV antigen cpm)/(mock antigen cpm), with error bars showing the standard deviation. Samples with two or more points in the dilution series giving stimulation indices of >2 have been taken to show positive stimulation by MVV antigen (+). The day p.i. is shown on the *x* axis, with individual sheep numbers indicated.

(Fig. 1), and all postinfection samples were positive for MVV DNA by PCR on cellular DNA (data not shown). This indicates that viral replication was occurring in all nodes by 4 days after infection. The frequency of infected cells, measured by an infectious center assay, was maximal between days 7 and 14 p.i. and was reduced by day 18 p.i., but the maximum frequency of cells with infectious MVV was only 23 $TCID_{50}$ per 10^6 total lymph node cells (day 14 p.i.). When lymph node sections were stained for *gag* p15 antigen-positive cells, a maximum of only one to two such cells were seen per field (×100 magnification), again suggesting a low frequency of productively infected cells.

Specific cellular immune responses to MVV antigen. The development of the cellular immune response to MVV was studied by using a cytotoxic assay predominantly measuring $CD8^+$ lymphocytes (5) and a T-cell proliferation assay.

The development of the CTL response was studied by assaying both for active CTL not requiring secondary in vitro stimulation and for precursor CTL (CTLp) activated by a 2-week culture with MVV antigen and IL-2. CTLp were detected as early as day 10 p.i. (one of two sheep) and then consistently after day 12 p.i. (Fig. 2A). Cytotoxicity was mediated by $CD8^+$ lymphocytes, as activity was lost when these cells were removed by complement lysis (data not shown). This reactivity was specific for strain EV1 MVV, the strain used to infect the sheep, CTLp activity was never seen against strain 1514 MVV, an Icelandic isolate which has been passaged many times in vitro (data not shown). In addition, CTLp activity was not detected 14 days after UV-inactivated virus challenge. At no time post-MVV infection were CTL which were active detected directly from the node (Fig. 2B).

Within efferent lymph samples from acutely infected nodes, only one of five sheep showed CTL activity without culture (4). We have since gone back to frozen efferent lymph cell samples and looked for the presence of CTLp. They were first detected leaving a prefemoral node between days 9 and 13 p.i. (>13% autologous compared with <6.5% heterologous MVV specific cytotoxicity) and leaving a popliteal node on day 6 (18.6% autologous compared with −6.4% heterologous MVV specific cytotoxicity).

MVV-specific proliferation by the lymph node cells was first detected on day 7 p.i. (both sheep showed positive proliferative responses) (Fig. 3). This response was then lost until day 12 to 14 p.i. (one sheep at each time point was positive) and was consistently seen after day 18 p.i. Sheep-to-sheep variation (both in their immune response genes and in the ability of virus to replicate) may have affected the time at which proliferation was seen for each individual. This proliferative assay has been shown to predominantly measure the $CD4^+$ lymphocyte-mediated response (33).

Infectious MVV (as detected by cocultivation) was therefore seen in the lymph node cell fraction before either detectable proliferative or CTL responses (MVV detected by day 4 p.i.). MVV-infected cells were also detected when there was a good proliferative and CTLp response (for example, one sheep at day 14 p.i. showed MVV-specific stimulation indices of greater than 40 and MVV specific cytotoxicity of greater than 40% and had 9 $TCID_{50}$ in 10^6 cells), although the maximal titers of infected cells were seen in a node at 14 days p.i., when there was a poor cytotoxic response and no detectable proliferative response.

Lymph node size and histological changes. From day 12 onwards, all acutely infected nodes were heavier than any control node (control group median, 0.83 g; range, 0.74 to 1.30 g) except for one node on day 18 p.i. The largest lymph nodes were removed on day 12 p.i., with a median weight of 2.85 g. Histological examination of sections of MVV-infected nodes showed changes indicative of a vigorous immune response, and the timing and intensity of some of the major features of this response are summarized in Table 2. Three components of the response were readily identified. First, activated lymphocytes were particularly prominent in the T-cell-rich paracortical area (T zone) adjacent to the intranodal lymphatics between days 4 and 18 p.i. These were identified by their large nuclei with open chromatin, the presence of active nucleoli, and abundant rRNA and mRNA, indicated by strong pyroninophilia in the nucleoli and cytoplasm of these cells (Fig. 4A). Second, plasma cells were seen in the medullary cords (Fig. 4B) but only from days 7 to 18 p.i., which coincides with the timing of the T-zone

TABLE 2. Histology of acutely MVV-infected lymph nodes

Sheep no.	Day p.i.	T-zone reaction[a]	Plasma cells in medullary cords[b]	Germinal center reaction[c]
1138W	0	−	−	−
1212T	4	+	+	+
1127W	4	+	+	+
1235T	7	++	++	Early
678R	7	End stage	++	−
1112W	10	−	+/−	++
1119W	10	++	++	++
1215V	12	−	+/−	+/−
1245T	12	++	++	++
1250T	14	−	+/−	+/−
1254T	14	+++	+++	+++
1219V	18	++	++	++ or −
1131W	18	++	++	+++
1238T	23	−	+	+ or −
573V	23	−	+/−	+++
1107W	40	−	−	+++
663R	40	−	−	++
1091P	630	−	−	+/−
1078P	534	+	+	+
1071P	599	−	−	−
638R rhs[d]	7	+	+	++
638R lhs[d]	14	+	+	+ or −

[a] Within the outer T-cell-rich zone, the lymphoblast response was scored as: −, quiescent; +, a few blasts detectable; ++, many blasts detectable; or +++, intensive blast response.

[b] Medullary cords contained: −, no or very occasional; +, relatively few; ++, many; or +++, confluent plasma cells.

[c] The follicles contained: −, no; +, small; ++, well developed; or +++, extensive germinal centers.

[d] UV-inactivated MVV controls.

blast reaction. Frequent cells which had a pyroninophilic cytoplasm characteristic of plasma cells were seen undergoing apoptosis in the medulla, suggesting that these plasma cells were short-lived. Apoptotic nuclear fragments were also found in macrophages of the medullary cords when plasma cells were present (Fig. 4B). Third, vigorous germinal center reactions occurred from day 10 and were still present at day 40 p.i. By immunohistology, there was no evidence that MVV antigen (*gag*) was trapped on the follicular dendritic cell network within germinal centers, as is seen with HIV and SIV (data not shown).

Phenotypic changes on lymph node cells. Lymph nodes contain a variety of mononuclear cell types: macrophages, interdigitating cells, and follicular dendritic cells as well as lymphocyte populations. Approximately 3% of cells were $CD1^+$ large cells and were considered to represent antigen-presenting interdigitating cells, while 5% of cells were $CD11c^+$ (CR4) and $CD14^+$ (lipopolysaccharide receptor) macrophage-like cells in control uninfected sheep, as shown in Table 3. At no time point postinfection did the percentage of these cell subsets alter significantly from those in uninfected controls.

In efferent lymph draining acutely MVV-infected nodes, one of the major features of the response to MVV was a rise in the percentage of large lymphoblasts, in particular, a marked rise in the percentage of $CD8^+$ lymphoblasts exiting the node between days 6 and 11 p.i., with the timing of this response varying among the sheep (4). To compare the node data with that seen in efferent lymph, the percentage of CD4, CD8, and B cells was analyzed in both small and large lymphocyte boxes defined by their FSC/SSC profile on FACS analysis (Table 3 and Fig. 5). No significant increase in the percentage of large cells in the node was detected following MVV infection, and only at day 12 p.i. did both sheep nodes have a significantly raised percentage of $CD8^+$ lymphoblasts (median, 15.5%) compared with uninfected nodes (median, 5.4%) (Fig. 5B). The percentage of $CD8^+$ small lymphocytes was slightly but not significantly raised from that at day 12 p.i. (data not shown). The rise in $CD8^+$ lymphocytes within the node is therefore much less marked and later than that seen in draining efferent lymph following MVV infection.

The $CD8^+$ lymphoblasts in efferent lymph had a very distinctive IL-2 receptor-negative ($IL\text{-}2R^-$), MHC class II DR and DQ profile (moderate DR with low DQ expression) suggestive of a homogeneous activation state. No IL-2R was ever detected on $CD8^+$ lymphocytes within the node. The MHC class II antigen expression of large and small $CD8^+$ cells for a sheep node with a high percentage of these $CD8^+$ cells at day 12 p.i. is shown in Fig. 5C and D. The DR and DQ antigen expression was always very heterogeneous on large $CD8^+$ lymphocytes, and although it was more homogeneous on $CD8^+$ small lymphocytes, the level of expression of both isotypes is greatly reduced compared with that seen on the large $CD8^+$ cells leaving the node via efferent lymph (4).

The percentage of $CD4^+$ and γδ T-cell receptor-positive (as measured phenotypically by T19 antigen expression) lymphocytes did not show any significant alterations with time p.i. In contrast, two sheep injected with UV-inactivated MVV showed a rise in the percentage of small and large $CD4^+$ cells in the node over the first 14 days (52.5 and 34.6%, respectively, on day 14). The percentage of B cells in infected nodes was not significantly different from that in uninfected nodes, but the control nodes showed extremely variable B-cell numbers, making significant changes unlikely.

DISCUSSION

The developing immune response to MVV within the lymph node draining the site of infection is described here and related to the recovery of infectious virus. This extends our previous studies on this relationship in draining efferent lymph from acutely infected nodes (4). Draining efferent lymph contains cells and factors that disseminate the response systemically, but it is within the lymph node that the major initial interaction of virus with the cells of the immune system occurs.

All the nodes from infected sheep had evidence of viral replication, but the frequency of virus-positive cells assessed by cocultivation was low (maximum, 23 $TCID_{50}$ in 10^6 cells). Only a small proportion of the MVV-susceptible cell lineages (13–15, 30) within the node were therefore infected, as macrophages and dendritic cells made up 1 to 8% of the isolated lymph node cells. Lentiviruses are known to exist in cells in vivo in highly restricted replication states (6, 19), and virus cocultivation assays may detect only a proportion of MVV-infected cells. Despite their low frequency, the number of infectious cells clearly peaked between days 7 and 14 p.i. After this time, the frequency of infectious cells dropped 10-fold, although MVV-positive cells could always be detected, confirming that the infection was never completely cleared from the initial draining lymph node.

It is difficult to compare the frequency of virus-infected cells from cocultivation assays with data for other lentiviruses, such as SIV and FIV, for which most information comes from in situ hybridization studies. In lymph nodes from SIV- or FIV-infected animals (within 3 months p.i.), many sections are negative for virus-infected cells by both antigen staining and in situ hybridization, but may rarely have up to 40 infected cells per field (3, 32, 34, 38). These frequencies do not seem to be very different from those seen in the studies of MVV-infected sheep

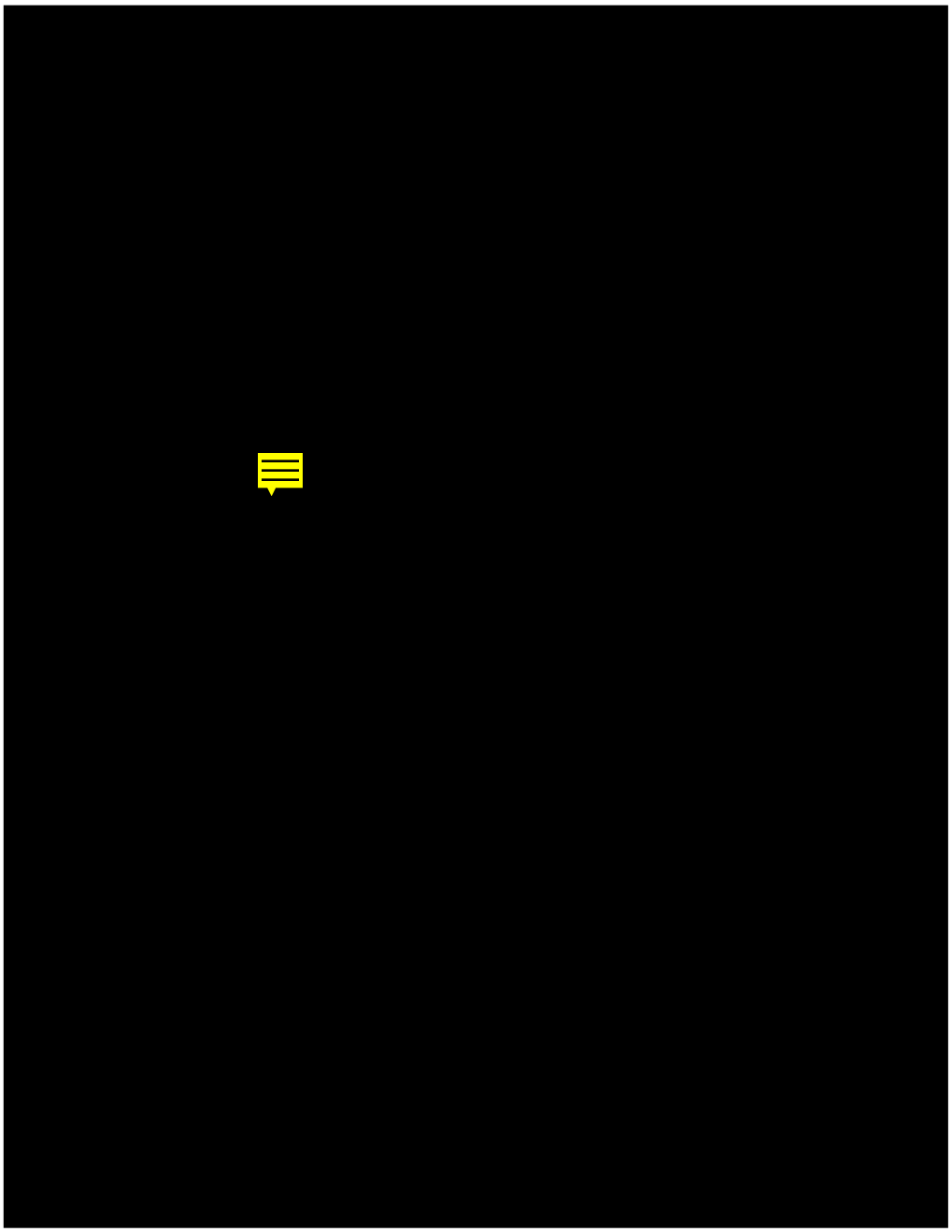

FIG. 4. Histological appearance of a prefemoral lymph node (sheep 1254T) at 14 days after local infection (methyl green pyronin staining; DNA blue-green with methyl green, and RNA crimson red with pyronin; magnification, ×500). (A) Section through the T-cell-rich zone. Several large pyroninophilic blast cells are present; three of these are indicated by arrows. (B) Section through a medullary cord which is full of intensely pyroninophilic plasma cells. The cytoplasm of macrophages in this area is almost unstained, and the strongly stained apoptotic (tingible) bodies (indicated by arrows) are easily seen against this pale background.

reported here and previously (37), especially as there is no lymphocyte involvement in MVV infection.

Within efferent lymph draining acutely MVV-infected lymph nodes, low frequencies of virus-infected cells were also seen by cocultivation which peaked on days 9 to 14 p.i. and then declined (4). The periods of maximal MVV infection in the node and efferent lymph are therefore very similar. The frequency of infectious cells is also very similar to that seen in draining efferent lymph, which is surprising considering that the major cellular targets for infection by MVV (macrophages and probably dendritic cells) are very rare in efferent lymph compared with the numbers found in lymph nodes. The proportion of macrophages (or dendritic cells) infected in efferent lymph must therefore be higher than that within the node, suggesting that altered migration of these infected cells occurs, allowing increased dissemination of the virus.

The periods of MVV infection in efferent lymph and lymph nodes are coincident with a specific immune response. Antibody to MVV *gag* p25 and *env* can be detected in draining efferent lymph from as early as day 4 p.i. (4), and this corre-

TABLE 3. Phenotype of cells recovered from control uninfected and persistently MVV-infected lymph nodes

Sheep[a]	% of total cells					% of small lymphocytes			% of lymphoblasts		
	$CD4^+$	$CD8^+$	$CD1^+$	$CD11c^+$	Large cells	$CD4^+$	$CD8^+$	Pan-B $cell^+$	$CD4^+$	$CD8^+$	Pan-B $cell^+$
Control											
Median	37.1	9.4	3.2	4.8	14.8	39.6	14.0	34.4	22.0	5.4	36.5
Range	15.3–40.2	2.7–14.5	1.2–5.5	1.5–8.0	4.0–22.0	18.0–47.0	4.0–18.0	20.0–65.0	8.0–36.0	2.0–8.0	27.0–55.0
Infected											
Median	43.0	15.5	4.4	7.3	13.0	50.9	17.8	19.4	23.3	11.4	25.6
Range	38.9–54.0	11.5–20.0	2.6–6.0	5.0–8.0	11.0–16.0	42.0–59.0	15.0–23.0	11.7–29.5	10.0–32.0	4.0–12.0	24.1–61.3

[a] Prefemoral lymph nodes from seven uninfected control sheep and persistently infected sheep 1091P, 1078P, and 1071P (see Table 2) were analyzed.

sponds to the time when plasma cells first appear in the medullary cords of the nodes. The humoral immune response may therefore contribute to the local containment of MVV in the lymph nodes of sheep. However, we were unable to detect MVV-specific antibody-producing cells in vitro by either p25 *gag* enzyme-linked immunosorbent spot assays (ELISA) or ELISA and Western (immunoblot) analysis of supernatant from cultured lymph node cells (data not shown). MVV-specific T-cell proliferative responses were detected within lymph node cells weakly on day 7 and consistently from day 18 p.i., while these responses were not seen until day 15 to 18 p.i. in efferent lymph (4). Proliferation to MVV antigen may not have been seen early with efferent lymph cells, as these showed a period of unresponsiveness to mitogen, concanavalin A, and IL-2. There was no evidence within the node for a period of unresponsiveness to IL-2 or mitogenic stimulation with concanavalin A or phytohemagglutin (data not shown), but there was a period, days 10 to 14, when proliferation to MVV was lost, and in both the node and its draining lymphatic, this was the period when the number of infected cells was increasing. CTLp were also detected during the period when infected cells were seen (from day 10 p.i.). However, virus-infected cell levels decreased and were maintained at low levels after the development of the specific immune response to MVV, suggesting that immunity does play a strong role in limiting viral load.

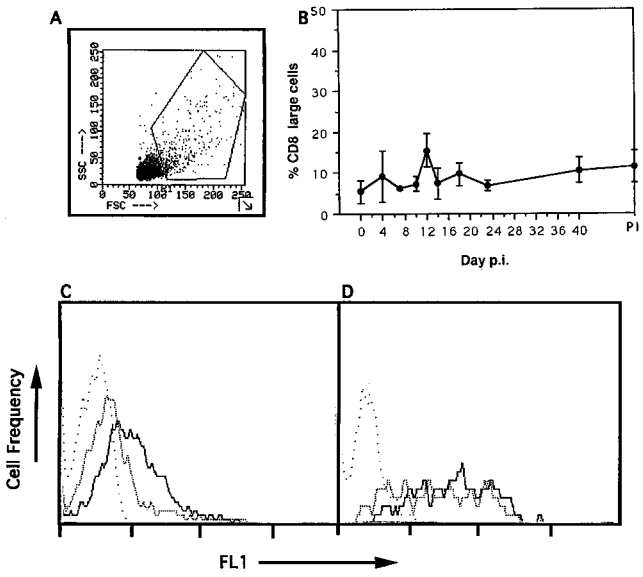

FIG. 5. Kinetics of appearance and MHC class II expression of large $CD8^+$ lymphocytes isolated from MVV-infected lymph nodes. (A) FSC/SSC profile of cells recovered from a day 12 infected lymph node, showing the box used to analyze large lymphocytes. (B) Levels of $CD8^+$ large lymphocytes determined by immunostaining and FACS analysis. Median and range are given for each time point. Seven uninfected nodes were analyzed at time zero, and two infected nodes were analyzed at other time points. Three persistently infected (PI) nodes were from sheep infected for approximately 18 months (see Table 2). (C and D) MHC class II DR and DQ expression on small (FSC, 62 to 103; SSC, 6 to 42) (C) and large (FSC, 109 to 255; SSC, 0 to 80) (D) high-intensity (fluorescence channel 2 [FL2], 160 to 255) $CD8^+$ lymphocytes analyzed by double staining of cells from a day 12 infected lymph node with anti-CD8 and either normal mouse serum (····), anti-DR (——), or anti-DQ (····).

CTLp were detected leaving acutely infected lymph nodes on day 6 p.i. (popliteal) and days 9 to 13 p.i. (prefemoral). This corresponded for each node to the characteristic appearance of $CD8^+$ lymphoblasts in the draining lymphatic. It is likely that part of this $CD8^+$ lymphoblast response includes virus-specific cells, although there may also be a polyclonal expansion of $CD8^+$ lymphocytes. This is seen in the response to influenza virus, when many $CD8^+$ cells show active cytokine transcription, although not all are influenza virus specific (8). The time of appearance of CTLp within the lymph node and efferent lymph corresponds well with the appearance of CTLp to SIV in blood (4 to 6 days p.i.) (39) and also in lymph nodes (7 days p.i.) (32) after intravenous challenge with SIV. However, it is much earlier than the detection of CTLp in blood after subcutaneous infection with MVV, which did not occur until between 3 weeks and 3 months p.i. (5).

The percentage of $CD8^+$ lymphoblasts did not alter significantly within the node at the time when we previously detected release of $CD8^+$ lymphoblasts into efferent lymph, but there was up to a threefold increase in node weight and the appearance of blast cells in the T zones of the nodes. This suggests that there is a balanced increase in all lymphoblast numbers, and therefore selective migration of $CD8^+$ lymphoblasts at a particular activation state (which we detected as a specific IL-2R and MHC class II profile) into efferent lymph is occurring. In the mouse model of influenza virus infection, there was also no increase seen in the percentage of $CD8^+$ lymphocytes within the mediastinal lymph node (1). A rise in the percentage $CD8^+$ cells was only seen in the lung, where active virus replication was occurring. These data support our conclusion that after viral infections, specific $CD8^+$ lymphocytes rapidly leave the node.

It is interesting to try to relate the in vitro immune response and virus data to the histological events occurring within the node. Experiments in rodents show that blast reactions in the T zone are dependent on the continued availability of antigen to B cells and interdigitating cells (24). Free antigen is rapidly eliminated in the presence of antibody, and the administration of antigen to an animal with a high specific antibody titer only induces a T-zone response for 3 to 4 days (24). As anti-MVV antibody is present from day 4 onwards, it seems likely that the extensive blast reaction in the T zone between days 4 and 18 is driven by the production of MVV by infected macrophages or

interdigitating cells throughout this period and only subsides when the number of virus-infected cells diminishes (after day 14 p.i.). Although some of the viral antigen that has bound antibody will be taken up and destroyed by macrophages, some will become localized on follicular dendritic cells. While antigen on these cells is involved in the activation of T and B cells in follicles, it does not induce or maintain lymphocyte proliferation in T zones (24). In HIV infection, during which large amounts of antigen persist in the follicles, there is usually relatively little lymphocyte activation in T zones. The presence of plasma cells in the medullary cords of lymph nodes or the equivalent area of the spleen, the red pulp, is directly linked to recent T-cell-dependent B-cell activation in the adjacent T zones. Plasma cells were seen in the medulla of infected nodes between days 4 and 23 p.i. This is consistent with their being the direct progeny of B blasts in the T zone. The short life span of medullary cord plasma cells in rodents seems also to apply to sheep, for medullary cord plasma cells were only seen in the nodes showing a T-zone blast reaction or within 5 days after it ended, and apoptotic plasma cells were seen during this time. Germinal centers are also characteristic of T-cell-dependent antibody responses (21) and are sites of massive clonal expansion of B cells (22). Germinal centers are formed from a small proportion of the B blasts activated in the T zone. From the time of arrival of blasts in the follicle to the end of the germinal center reaction is about 3 weeks (24). The persistence of germinal centers for 3 weeks after the end of the T-zone reaction is consistent with the reported duration of germinal center responses in rodents. It therefore appears that these acutely infected lymph nodes show many of the typical histological responses of primary antigenic stimulation.

The data presented here and previously (4) show that within the first 14 days after infection with the lentivirus MVV, there was increasing virus replication within lymphoid tissue. During this time, a specific immune response was induced which showed many of the histological features seen in primary protein antigen responses and included a $CD8^+$ lymphoblast response containing MVV-specific CTLp. After this immune response was detected, the number of infected cells decreased and, histologically, antigen presentation to T cells appeared to cease. Despite this normal histological response, the MVV-specific proliferative response was not detectable between days 10 and 14, and this might allow sufficient virus replication and dissemination for persistence to be established. The continuing low frequency of MVV-positive cells detected in the nodes after day 14 p.i. is evidence for this lentiviral persistence. Once a reservoir of infected cells is established, restricted virus replication in vivo may then become an important immune evasion mechanism (6, 18). Parallel studies on these nodes to examine the extent of restricted virus replication within lymph node cells are in progress.

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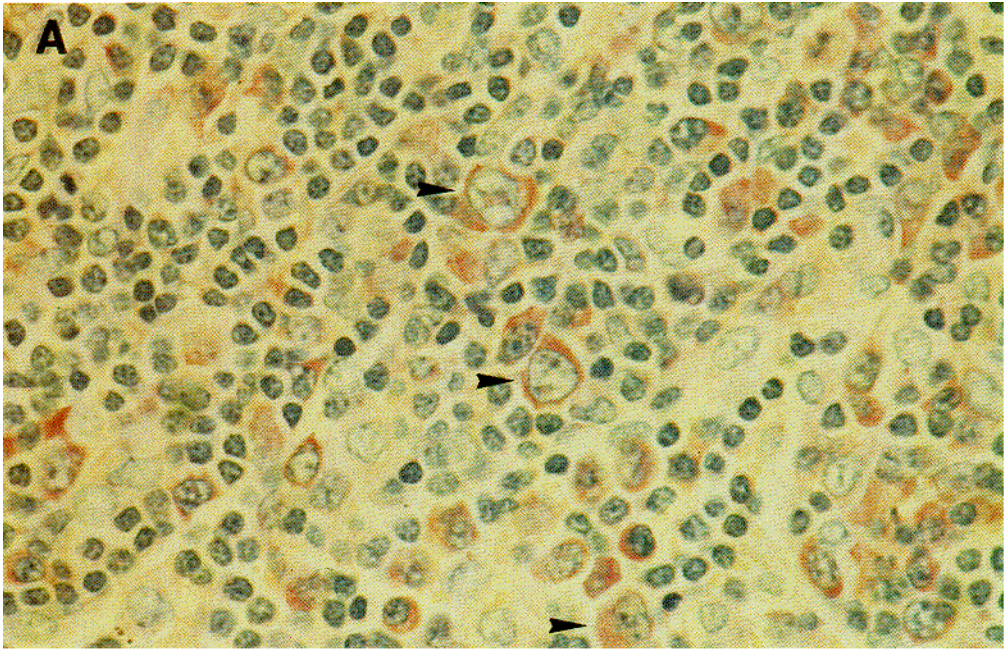
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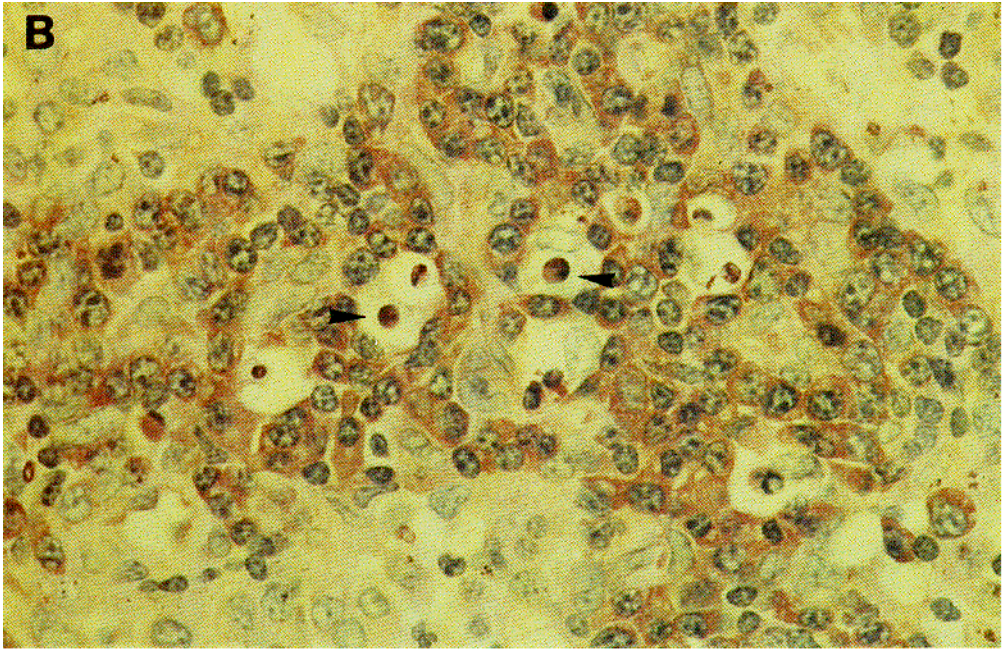
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