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Inflammation-induced changes in the phenotype and cytokine profile of cells migrating through skin and afferent lymph

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SUMMARY

In the present study, we have localized cytokine-secreting cells within an ectoparasite-induced inflammatory lesion and monitored the phenotype and cytokine profile of cells migrating from the inflammatory lesion to the local draining lymph node via the afferent lymphatics. Interleukin (IL)-8-producing cells were first detected in skin within 6 hr of infection, with increased numbers observed at 24 and 48 hr post infection. While these cells were concentrated within the neutrophil influx, adjacent to disrupted epidermis; they were also found scattered throughout the surrounding dermis in areas where significant cellular infiltration was not apparent. IL-1α- and IL-1β-producing cells could not be detected until 24 hr after infection and were restricted to areas of intense neutrophil accumulation. Concurrent with the onset of inflammation was a threefold increase in the total number of cells migrating through the draining afferent lymph. This increase in cellularity was due primarily to increased migration of CD4 and $\gamma\delta$ T cells. Cytokine mRNA synthesis by migrating afferent lymph cells was examined by reverse transcription–polymerase chain reaction (RT–PCR) analysis of RNA extracted prior to, and at regular intervals during the course of the inflammatory response. IL-1β and IL-8, but not IL-1α or IL-6 mRNA, was detected in migrating afferent lymph cells. Tumour necrosis factor (TNF)-α-specific mRNA was present in migrating afferent lymph cells at all time points both prior to, and following infection. Soluble IL-8 protein, but not IL-1α, IL-1β or TNF-α protein, could be detected in lymph, with the amount of IL-8 detected increasing as the infection progressed. mRNA coding for cytokines associated with T-cell activation, such as IL-2, IL-4 or interferon (IFN)-γ, was also detected in migrating cells, although the cytokine profiles of different experimental animals were extremely variable.

INTRODUCTION

Development of host immunity to infection is dependent on cellular migration between different anatomical compartments. Foreign antigens are usually acquired through epithelial surfaces, such as skin, gut or lung, with the development of specific immunity occurring primarily within draining lymphoid tissue where specialized endothelium permits the extraction of large numbers of naive T cells from blood.[1] While the uptake and transport of antigen from the periphery to draining lymphoid tissue by dendritic cells has been the subject of intense study,[2] the potential of other peripheral responses, such as localized acute inflammation, to impact on the concurrent development of specific immunity is less well understood. A number of studies have demonstrated that cutaneous inflammation induced by chemical irritants, contact hypersensitivity or ultraviolet irradiation results in the enhanced migration of dendritic cells to draining lymph nodes.[3,4] Clearly, however, there are further mechanisms whereby localized inflammation might impact on the development of immunity in draining lymphoid tissue. Various mediators of acute inflammation, such as interleukin (IL)-1, tumour necrosis factor (TNF)-α and IL-6 are also intimately involved in the development of specific immunity[5–8] and cells producing these cytokines could potentially be transported from inflammatory tissue in the periphery to the draining lymph node within afferent lymph. In addition, the preferential extraction by inflammatory endothelium of T cells expressing predominantly a memory phenotype,[9] and the traffic of these cells through inflamed tissue back to the draining lymph node, via the afferent lymphatics,[10] may also influence the cytokine profile within draining lymphoid tissue. However, analysis of the cellular and cytokine profile of afferent lymph and changes to this profile induced by localized inflammation has been very limited.

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; mAb, monoclonal antibodies; RT, reverse transcription; TCR, T-cell receptor; TNF, tumour necrosis factor.

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To address these issues we have cannulated sheep 'pseudo-afferent' lymph ducts and monitored changes in the cellular and cytokine profile of afferent lymph during the onset of acute cutaneous inflammation induced by ectoparasite (*Lucilia cuprina*) infection. These changes were correlated with changes in the synthesis of cytokine proteins within the inflammatory lesion. The response within skin to infection with this ectoparasite has previously been characterized in terms of both infiltrating cells and local cytokine mRNA synthesis.[11,12] Cellular infiltration is initiated within 6 hr of infection and by 48 hr is characterized by an intense accumulation of neutrophils into the upper dermis and of macrophages, eosinophils and CD4 and $\gamma\delta$ T cells into the mid-dermis.[11] This infiltration correlates with increased production of mRNA encoding various inflammatory cytokines including IL-1α, IL-1β, IL-6 and IL-8, as well as a number of cytokines associated with T cell activation such as IL-2, IL-4 and IFN-γ.[11,12] The results presented in this study extend our previous observations to demonstrate the impact that an acute inflammatory insult in the skin can have on both the cellular and cytokine profiles of the draining afferent lymph, and thus potentially on the development and differentiation of the immune response in the draining lymphoid tissue.

MATERIALS AND METHODS

Experimental animals and surgical procedures

Two to three-month-old merino lambs, not previously exposed to ectoparasite infection, were maintained at the School of Veterinary Science, The University of Melbourne, on a diet of lucerne chaff and pellets. Eight weeks prior to cannulation, the prefemoral lymph nodes were surgically removed and the animals were rested for at least 6 weeks to allow the lymph ducts to re-anastomose. The resulting 'pseudoafferent' lymph duct was cannulated as previously described.[13] Lymph was collected into sterile plastic bottles containing 100 U heparin, 500 U penicillin and 0·1 mg streptomycin (CSL Ltd, Parkville, Australia). Sheep were rested for at least 2 days following surgery, prior to infection.

Experimental infections

Lucilia cuprina eggs were obtained from Dr M. Sandeman, Department of Agriculture, LaTrobe University (Melbourne, Australia), and were allowed to hatch overnight at 37°. For immunohistological analysis of infected skin, $\approx$200 newly hatched larvae were placed on two to three sites on the flank of the sheep using a standard implant technique.[14] Biopsy specimens were taken from infection sites at regular intervals following infection, embedded in OCT medium (Tissue Tek, Miles, Ekhart, IN) and frozen in liquid nitrogen. For infection of cannulated sheep, larvae were placed on the flank of the sheep at two sites drained by the cannulated lymph duct and the infections allowed to proceed for 48 hr before the larvae were removed. Afferent lymph was sampled at regular intervals over the course of infection and cells were recovered from lymph fluid by centrifugation. Lymph fluid was stored at $-20°$.

Monoclonal antibodies

Monoclonal antibodies (mAb) specific for ovine cell surface makers were 1.28 (CD45), 44.97 (CD4), 38.65 (CD8), 20.27 (CD1), 20.96 (CD45R), 86D [$\gamma\delta$ T-cell receptor (TCR)][12] and 9.14 [IL-2 receptor (R)-α chain].[15] Monoclonal Ab specific for ovine cytokines were 10.82 (IL-1α), 3.41 and CSIRO-1β (IL-1β),[16] 6.09/TNF3 (TNF-α),[17] 8M6 (IL-8) and 8D8 [granulocyte–macrophage colony-stimulating factor (GM-CSF), gift from Dr C. McInnes, Moredun Research Institute, Edinburgh]. Monoclonal Ab 21.51, raised against ovine growth hormone, was used in all experiments as an isotype-matched negative control.

Flow cytometric and immunohistological analysis of cellular phenotype and cytokine synthesis

Afferent lymph cells were stained for flow cytometric analysis every 6–8 hr over the course of infection as previously described.[15] Cellular fluorescence was analysed on a fluorescence activated cell analyser (FACScan, Becton Dickinson, Mountain View, CA). For immunohistology, 5 μm cryostat tissue sections were cut from skin biopsies, mounted on glass slides and fixed in cold ($-20°$) acetone for 5 min. Sections were stained using an indirect alkaline phosphatase–anti-alkaline phosphatase technique.[18]

RNA extraction and detection of cytokine-specific mRNA by RT–PCR

RNA was extracted from afferent lymph cells every 4–6 hr following infection of sheep with *L. cuprina* larvae, as previously described.[19] First strand cDNA was prepared by incubating 5 μg total RNA with 10 μl 5$\times$ reverse transcriptase buffer (Promega, Madison, WI), 4 μl of 0·1 M dithiothreitol, 1 μl RNAse inhibitors (Boehringer Mannheim, Mannheim, Germany), 5 μl of 10 mM dNTPs, 2 μl random decamers (150 ng/μl) and 20 U MMLV reverse transcriptase (Promega). The reaction mixtures were made up to 50 μl with DEPC treated water and incubated for 1 hr at 37°. The PCR mixtures comprised 2·5 μl 10$\times$ Taq buffer (Perkin Elmer, Branchburg, NJ), 1 μl 5 mM dNTPs, 0·5 μl each of forward and reverse primers (100 ng/ml), 0·5 μl Taq polymerase (Perkin Elmer) and 2·5 μl cDNA. The reactions were made up to 25 μl with H_2O and overlaid with 30 μl mineral oil. PCR amplification was carried out for 35 cycles of denaturation at 94° for 1 min, annealing at 55° for 1 min and amplification at 72° for 1 min. Sequences for the forward and reverse primers specific for each mRNA and the expected fragment sizes in base pairs (bp) were as follows: IL-1α-GCTTCAAGGAGAATGTGG, GAGAATCCTCTTCTGATAC, 338 bp; IL-1β-TACAGTGATGAGAATGAG, TCTCTGTCCTG-GAGTTTG, 335 bp; IL-2-AACTCTTGTCTTGCATTG, GATG-CTTTGACAAAAGGT, 436 bp; IL-4-TGCATTGTTAGCGTCT-CC, TATTCAGCTTCAACACT, 470 bp; IL-6-GCTTCCAATC-TGGGTTCA, CCACAATCATGGGAGCCG, 347 bp; IL-8-ATG-AGTACAGAACTTCGA, TCATGGATCTTGCTTCTC, 222 bp; IL-10-AGCTGTACCCACTTCCCA, GAAAACGATGACAGC-GCC, 305 bp; TNFα-GGCTCTCCTGTCTCCCGT, GTTGGCT-ACAACGTGGGC, 335 bp; IFNγ-ATGGCCAGGGCCCATTTT, ATTGATGGCTTTGCGCTG, 338 bp; GM-CSF-ATGTGGCTG-CAGAACCTGCTTCTC, CCTCTGGGCTGGTTCCCAGCAGTC, 438 bp; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-ATCACTGCCACCCAGAAGACT, TTGCCCTTCGAGTGACC-GTAC, 152 bp.

Five microlitres of product from each PCR reaction was electrophoresed on a 1·5% agarose gel and Southern blotted onto a Hybond-N+ membrane (Amersham, North Ryde, NSW, Australia) in 10$\times$ SSC. Specific cDNA probes for each cytokine were radiolabelled by random priming in the presence of 50 μCi α^{32}P-dCTP (ICN Biomedicals, Seven Hills, NSW, Australia), using a GIGAprime DNA labelling kit according to the manufacturer's instructions (Bresatec, Thebarton, SA, Australia). Filters were

hybridized with the probes at 65° overnight, washed under conditions of high stringency (0·1× SSC, 0·5% sodium dodecyl sulphate (SDS), 65°) and autoradiographed.

Cytokine-specific immunoassays

The ovine IL-1α, IL-1β and TNF-α-specific immunoassays were carried out as previously described[16,17] using ascites of mAbs 10.82 (1 : 250) CSIRO-1β (1 : 500) and 6.09/TNF3 (1 : 250), respectively. An identical protocol was used for the ovine IL-8-specific immunoassay after microtitre trays were coated with 50 μl/well of a 5 μg/ml solution of purified mAb 8M6, diluted in carbonate buffer (35 mM $NaHCO_3$, 5 mM Na_2CO_3, 0·2% NaN_3, pH 9·6).

RESULTS

Changes in the cellular phenotype of skin during parasite-induced acute inflammation

Sheep were infected with *L. cuprina* larvae and skin biopsies taken at regular intervals over the course of the infection for immunohistological analysis. After 48 hr of infection there was an almost complete disruption of the epidermis and substantial infiltration of cells expressing CD45 (predominantly neutrophils) into the upper dermis of the skin (Fig. 1a and b). This infiltration of neutrophils was first apparent within 6 hr of infection and was well-established by 24 hr. Significant infiltrations of both CD4 and $\gamma\delta$ T cells were seen in the mid-dermis at 24 hr after infection, with numbers increasing as the infection progressed (not shown). Staining for IL-2Rα chain expression on serial sections indicated that the vast majority of infiltrating T cells expressed this marker, indicating recent activation (Fig. 1c and d). Very few CD8 T cells or $CD45R^+$ B cells were detected in either normal or infected skin at the time points examined. After 48 hr of infection, an increase in the number of macrophages and $CD1^+$ Langerhans' cells was seen in the upper dermis, below the layer of neutrophil accumulation, and increased numbers of eosinophils were detected in the lower dermis (not shown).

Immunohistological analysis of cytokine expression in skin during parasite-induced acute inflammation

Frozen sections from skin biopsies taken from infected and uninfected sites were assessed for the presence of cytokine secreting cells (IL-1α, IL-1β, TNF-α, IL-8 and GM-CSF) by immunohistology. No cytokine-specific staining was seen in skin sections taken from uninfected sites (not shown). After 6 hr infection, IL-8-specific staining was detected within the inflammatory lesion and correlated with the presence of neutrophils infiltrating into the epidermis at this early time point. No IL-1α or IL-1β-specific staining was detected at this time. By 24 hr after infection, IL-8-specific staining was most intense within the area of neutrophil accumulation adjacent to the disrupted epidermis. Cells staining positive for IL-1α and IL-1β were first detected at this time and were also confined to the areas of intense neutrophil accumulation near the disrupted epidermis. Cytokine-specific staining in skin after 48 hr infection is shown in Fig. 2. IL-8 staining was most intense within the layer of neutrophil infiltration adjacent to the disrupted epidermis at the site of infection, although IL-8-specific staining also extended into the undisrupted epidermis where there was, as yet, little neutrophil infiltration. In contrast, discreet clusters of IL-1β positive cells were confined to an area within the neutrophil accumulation and were not found elsewhere within the epidermis. A similar pattern of staining was seen for IL-1α, although IL-1α positive cells were not as numerous as those positive for IL-1β. Very few TNF-α positive cells were detected in infected skin and no GM-CSF-specific staining was observed. No cytokine-specific staining was detected within the areas of macrophage or lymphocyte accumulation in the mid-dermis.

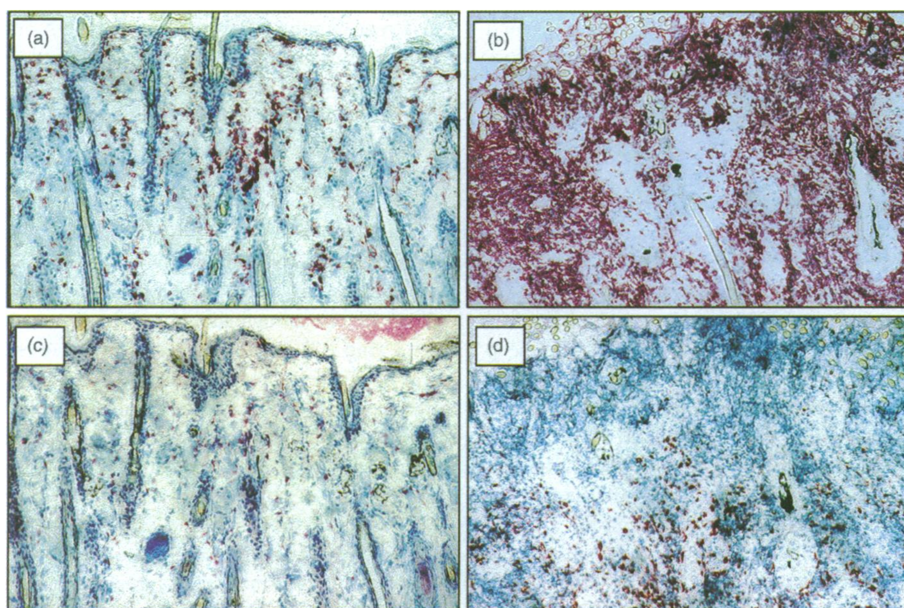

Figure 1. Immunohistological analysis of ovine leucocyte subsets in normal and inflamed skin. Frozen sections of biopsies taken from normal skin (a and c) or from skin infected for 48 hr with ≈200 newly hatched *L. cuprina* larvae (b and d) were stained with mAbs specific for ovine CD45 (a and b) and the IL-2Rα chain (c and d). Magnification ×100.

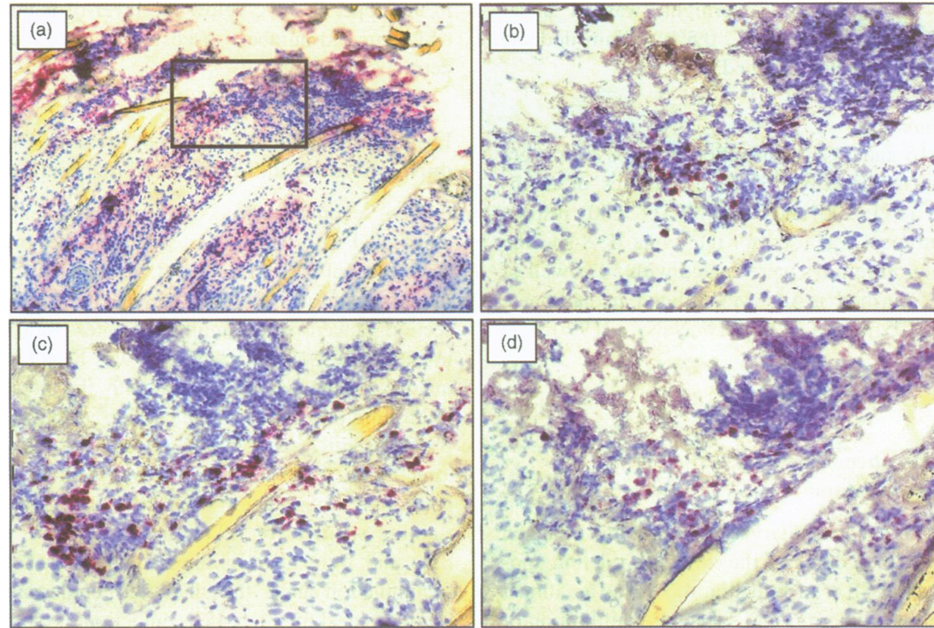

Figure 2. Immunohistological analysis of ovine cytokine expression in inflamed skin. Frozen serial sections of biopsies taken from skin infected for 48 hr with ≈200 newly hatched *L. cuprina* larvae were stained with mAbs specific for ovine CD45 (a), IL-1α (b), IL-1β (c) and IL-8 (d). Cytokine-specific staining is shown for the area contained within the box in a. Magnification ×100 (a) and ×400 (b, c and d).

Tissue staining was specific for each cytokine, as pre-incubation of the mAbs with an excess of the appropriate recombinant cytokine blocked staining of the sections (not shown).

Phenotype of afferent lymph cells migrating from the site of parasite-induced acute inflammation

In all sheep examined, *L. cuprina* infection induced an increase in the total number of cells migrating through the afferent lymph (representative data, Fig. 3a). This increase in cellularity was first apparent within 12 hr of infection and peaked between 24 and 30 hr before declining. The magnitude of the increase ranged from approximately three- to sixfold in the six experimental sheep examined. Infection did not result in any significant change in the volume of afferent lymph draining from the site of infection.

To assess inflammation-induced changes in the phenotype of cells migrating through afferent lymph, afferent lymph cells were stained with a panel of mAbs reactive against various ovine leucocyte subsets for flow cytometric analysis (Fig. 3b). Infection resulted in an approximate fourfold increase in the number of CD4 T cells in draining afferent lymph. This increase, which peaked between 18 and 30 hr after infection, was seen in all sheep examined and ranged from two- to fourfold. A similar increase in the number of $\gamma\delta$ T cells migrating through the afferent lymph was also observed in five of six sheep examined. Both CD4 and $\gamma\delta$ T cells were predominantly positive for expression of IL-2Rα (not shown). In contrast, there was no significant change in the number of CD8 T cells or CD45R$^+$ B cells migrating through the afferent lymph. There was also no significant change in the number of migrating CD1$^+$ dendritic cells throughout the infection period. Migration of granulocytes, subsequently identified as neutrophils, was assessed by the distinct forward/side scatter profile of these cells. Two of the six sheep examined showed dramatic increases in the number of these cells migrating through lymph during the final 24 hr of the response. In these sheep, neutrophils increased from ≈1% of cells prior to infection, to up to 40% of afferent lymph cells at the peak of their output. It was noted that the presence of neutrophils in the afferent lymph correlated with the presence of erythrocytes in the lymph and the severity of the infection.

Expression of inflammatory cytokine-specific mRNA by afferent lymph cells during parasite-induced acute inflammation

Afferent lymph cell expression of mRNA coding for inflammatory cytokines was analysed both prior to and during infection. Total RNA was extracted from cells collected every 4–6 hr over the course of the infection and subjected to RT–PCR analysis using primers specific for each of a number of ovine cytokines. Southern hybridization of the PCR products to specific cDNA probes was used to confirm the specificity of the amplified product. In addition to cytokine mRNA, all RNA samples were analysed for the presence of mRNA coding for ovine GAPDH as a positive control. Analysis of six sheep was carried out with representative results from the one animal shown in Fig. 4.

Using RT–PCR analysis, no mRNA encoding IL-1α or IL-6 was detected in RNA extracted from afferent lymph cells of any of the sheep tested. In contrast, infection of sheep with *L. cuprina* larvae induced the expression of IL-1β-specific mRNA in afferent lymph cells migrating from the site of the infection (Fig. 4). In most cases, IL-1β-specific mRNA was first detected between 6 and 8 hr after infection and was usually present only within the first 24 hr of the response. Synthesis of ovine IL-8-specific mRNA was also induced in afferent lymph cells migrating from the site of *L. cuprina* infection in all sheep examined. In most cases, IL-8-specific mRNA was first detected ≈6 hr after initiation of infection and, in some cases, expression persisted for up to 72 hr. Ovine TNF-α-specific mRNA was detected in RNA extracted from

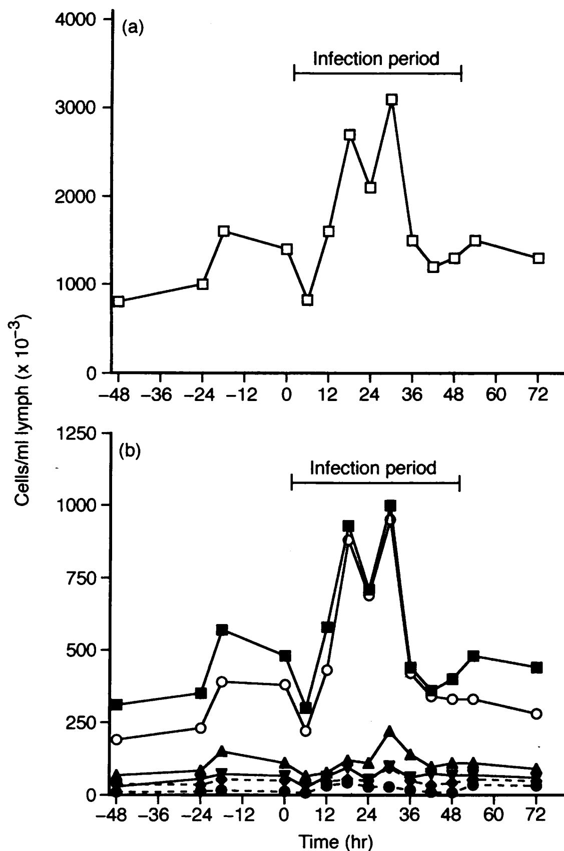

Figure 3. Cell output from a cannulated pseudoafferent lymphatic draining a site of parasite induced acute inflammation. Sheep were infected with ≈200 newly hatched *L. cuprina* larvae at two sites drained by the cannula for the indicated period. Lymph was collected at regular intervals prior to and following infection. (a) Total cell output. (b) Output of CD4 (■), CD8 (♦), γδ TCR (○), CD1 (▼) and CD45R (▲) positive lymphocytes and neutrophils (●).

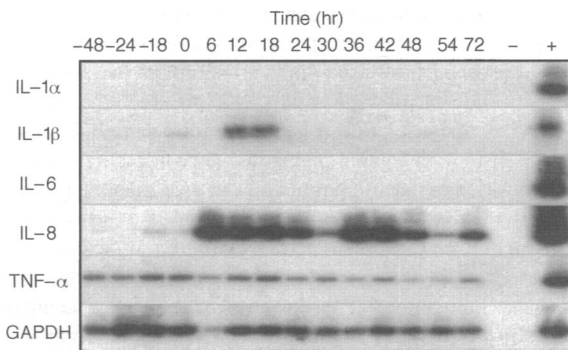

Figure 4. Detection of mRNA coding for inflammatory cytokines in RNA extracted from afferent lymph cells prior to and following induction of acute inflammation in the skin. Sheep were infected with ≈200 newly hatched *L. cuprina* larvae at two sites drained by the cannula. Total RNA was extracted from afferent lymph cells at the times indicated and cytokine-specific mRNA was detected following PCR amplification of cDNA prepared from afferent lymph cell RNA. The integrity of the cDNA was confirmed by amplification of PCR products using primers specific for ovine GAPDH. Cytokine-specific cDNA or unrelated cDNA were included as positive and negative controls for all PCR reactions as shown. Specificity of PCR products was confirmed by Southern hybridization of PCR products to ^{32}P-labelled cytokine-specific cDNA probes.

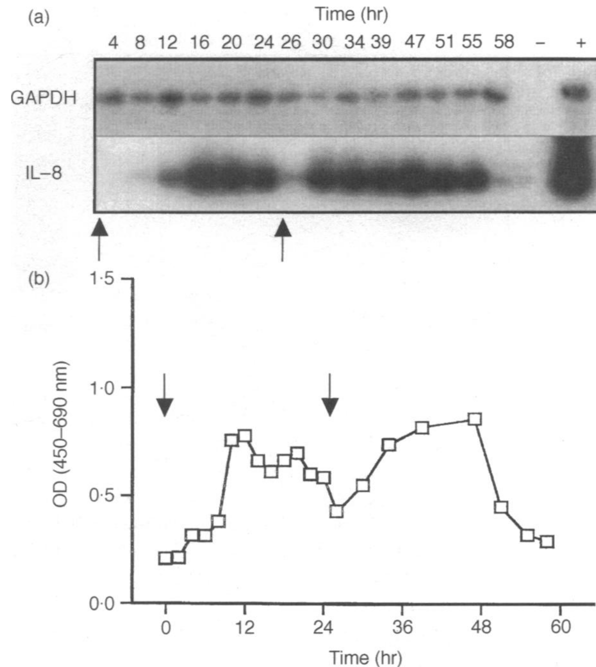

Figure 5. IL-8-specific mRNA and IL-8 protein in afferent lymph following sequential induction of acute inflammation. Sheep were infected with ≈200 newly hatched *L. cuprina* larvae at two sites drained by the cannula, 24 hr apart, as indicated by the arrows. Lymph was then collected from a cannulated duct at regular intervals following infection. (a) Total RNA was extracted from afferent lymph cells and cytokine-specific mRNA was detected following PCR amplification of cDNA prepared from afferent lymph cell RNA. Specificity of PCR products was confirmed by Southern blot analysis. (b) Cell-free lymph was prepared by centrifugation at 1500 *g* for 5 min and soluble IL-8 was determined using a specific immunoassay.

afferent lymph cells at all time points analysed, both before and after infection.

IL-8 protein in afferent lymph correlates with initiation of acute inflammation and the presence of IL-8-specific mRNA

As well as analysing inflammatory cytokine synthesis at the level of mRNA, immunoassays specific for ovine IL-1α, IL-1β, IL-8 and TNF-α were used to detect the presence of cytokine proteins in the afferent lymph following infection. No IL-1α, IL-1β or TNF-α was detected in the lymph at any time prior to or during infection with *L. cuprina* larvae, despite the presence of mRNA coding for IL-1β and TNF-α in cells migrating from the site of infection. The sensitivity of these immunoassays were ≈5 pg/ml for IL-1α, 10 pg/ml for IL-1β and 200 pg/ml for TNF-α, based on the detection of the corresponding recombinant cytokines.[16,17] In contrast, IL-8 was detected in the lymph of all sheep tested and correlated with the initiation of infection and the presence of specific mRNA in migrating afferent lymph cells. This is depicted in Fig. 5, where sequential infections were established, with a 24 hr interval, in an area drained by the one cannulated lymph duct. Following the first infection, IL-8-specific mRNA was detected within 8 hr, in total RNA extracted from the afferent lymph cells (Fig. 5a). By 24 hr after infection, however, the relative amount of IL-8-specific mRNA had clearly declined. Following initiation of the second infection, the relative amount of IL-8-specific mRNA

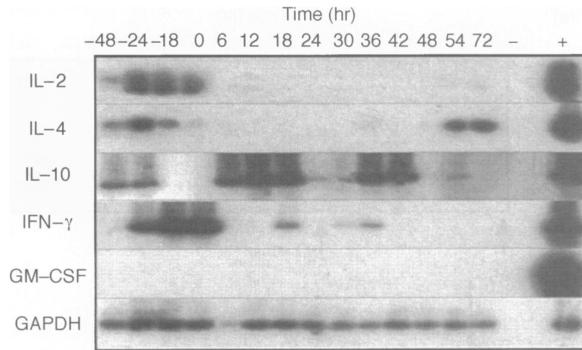

Figure 6. Detection of mRNA coding for cytokines associated with T cell activation in RNA extracted from afferent lymph cells prior to and following induction of acute inflammation in the skin. Sheep were infected with ≈200 newly hatched *L. cuprina* larvae at two sites drained by the cannula. Total RNA was extracted from afferent lymph cells at the times indicated and cytokine-specific mRNA was detected following PCR amplification of cDNA prepared from afferent lymph cell RNA. Specificity of PCR products was confirmed by Southern blot analysis.

detected in RNA extracted from afferent lymph cells again increased to peak ≈12 hr later before declining. A similar biphasic response was seen in the concentration of IL-8 protein in the lymph fluid (Fig. 5b). The amount of IL-8 in the lymph peaked 12 hr after the first infection, but had declined by 24 hr. Following re-infection the amount of IL-8 in the afferent lymph again increased, reaching a peak 24 hr later.

Expression of T-cell-associated cytokine-specific mRNA by afferent lymph cells during parasite-induced acute inflammation

In addition to inflammatory cytokine mRNA, total RNA extracted from afferent lymph cells prior to and during *L. cuprina* infection was analysed for the presence of mRNA coding for cytokines which are usually associated with T-cell activation. In contrast to the consistent pattern of expression seen for inflammatory cytokines in afferent lymph cells, the expression of T-cell associated cytokines was more variable. Analysis of afferent lymph for mRNA encoding T-cell associated cytokines from one sheep is shown in Fig. 6, while a summary of the expression of these cytokines by individual sheep examined is presented in Table 1. Ovine IL-4-specific mRNA was detected in total RNA extracted from afferent lymph cells recovered from five of six sheep analysed. Comparison of the expression profiles, however, revealed no relationship between the initiation of infection and induction of expression with, IL-4 mRNA detected prior to, and at a variety of time points during infection. Expression of IL-2 and IFN-γ mRNA by afferent lymph cells was similarly variable and no consistent pattern in the co-expression of these cytokines, either prior to, or following the initiation of inflammation was seen. Ovine IL-10-specific mRNA was detected in RNA extracted from afferent lymph cells of all sheep examined both prior to and during the infection. In some animals, IL-10-specific mRNA was detected in total RNA isolated at all time points both before and during the response to infection. Other sheep, however, showed discreet pulses of IL-10 mRNA expression (Fig. 6), reminiscent of IL-8 mRNA expression. No mRNA specific for GM-CSF was detected in RNA extracted from the afferent lymph cells of any of the sheep.

Table 1. Expression of mRNA coding for T-cell associated cytokines in afferent lymph cells prior to or following initiation of parasite-induced acute inflammation

		Cytokine*				
		IL-2	IL-4	IFN-γ	IL-10	GM-CSF
Sheep 1	Uninfected	+	+	+	+	−
	Infected	−	+	+	+	−
Sheep 2	Uninfected	−	+	−	−	−
	Infected	−	+	−	+	−
Sheep 3	Uninfected	+	+	+	+	−
	Infected	−	+	+	+	−
Sheep 4	Uninfected	−	−	−	+	−
	Infected	+	−	−	+	−
Sheep 5	Uninfected	+	+	−	+	−
	Infected	+	+	−	+	−
Sheep 6	Uninfected	−	+	−	+	−
	Infected	−	+	−	+	−

*Cytokine-specific mRNA was detected in RNA extracted from afferent lymph cells by RT–PCR and Southern hybridization to cytokine-specific cDNA probes.

DISCUSSION

Localized acute inflammation at the site of an infection has the potential to influence the development of immunity by altering the cellular composition and/or cytokine profile of afferent lymph draining to regional lymphoid tissue. This issue has been difficult to approach experimentally, however, primarily due to the technical difficulties associated with accessing the afferent lymph compartment in small animal models. Ruminants, particularly sheep, have been the primary large animal model for studies of lymphocyte recirculation through afferent or efferent lymphatics.[9,10,13] As a result of recent advances in ruminant cytokine biology,[20,21] we have been able to undertake studies to directly address the impact of acute inflammation on the cellular and cytokine profile of afferent lymph. Our results demonstrate that many of the changes induced in skin during acute inflammation, with respect to both cellular and cytokine profiles, are reflected in the composition of the afferent lymph and thus, presumably, within draining lymphoid tissue.

Production of the inflammatory cytokines IL-1α, IL-1β and IL-8 was detected following induction of inflammation, in cells located predominantly within the layer of infiltrating neutrophils adjacent to the disrupted epidermis. Activated neutrophils have previously been demonstrated to synthesize both IL-1 and IL-8[22] and there is extensive evidence supporting a role for both these cytokines in the initiation and progression of the inflammatory lesion.[23] While production of IL-8 was first detected within 6 hr of initiation of infection, IL-1α and IL-1β producing cells were not detected until 24 hr when the inflammatory lesion was well established. These kinetics correlated closely with those described for the synthesis of the respective cytokine-specific mRNA in infected skin.[12] mRNA coding for IL-8 was detected 6 hr after infection, and although increased levels of IL-1α and IL-1β-specific mRNA were detected at 6 hr post infection, the level of mRNA encoding these cytokines did not peak until 24 hr into the response.

Cellular migration from the site of inflammation to the draining lymph node was studied following cannulation of pseudoafferent lymph ducts. Following infection of sheep with *L. cuprina* larvae, there was a selective recruitment of IL-2Rα positive–CD4 and $\gamma\delta$ T cells into the skin, and a corresponding increase in migration of these cells through the afferent lymph. A similar correlation between infiltration of specific lymphocyte subsets into the skin and migration of the same subsets through draining afferent lymph was observed following cutaneous infection of cattle with *Trypanosoma congolense*. Infection with this parasite induced a localized skin reaction characterized by infiltration of B cells and CD4 and CD8, but not $\gamma\delta$, T cells.[24] These changes coincided with a marked decrease in the number of $\gamma\delta$ T cells and an increase in the number of B cells migrating through afferent lymph.[25] The acute inflammatory response to infection with *L. cuprina* larvae did not appear to significantly alter the migration of dendritic cells through afferent lymph. This also correlated with observations reported following infection of cattle with *T. congolense* but contrasts with reports describing enhanced migration of dendritic cells from inflammatory sites induced by non-infectious agents.[3,4] While this difference may simply reflect the destructive nature of the parasite-induced lesion, it may also be the result of subtle differences in the induction of local cytokine synthesis. For example, while TNF-α has been shown to induce the migration of Langerhans' cells from skin,[26] infection with *L. cuprina* larvae does not appear to modulate the amount of TNF-α-specific mRNA or protein.[12]

The cytokine profile of afferent lymph preceding and during induction of the inflammatory lesion was monitored by RT–PCR analysis of total RNA and immunoassay of cell-free lymph. The results correlated with previous analysis of inflammatory cytokine expression in the skin. For example, IL-6 mRNA in skin was localized to endothelium and fibroblast-like cells,[12] which do not recirculate through afferent lymph. The reason for the detection of IL-1β but not IL-1α mRNA is less clear. The kinetics of induction of both IL-1 mRNA species in skin early in the response were similar and while both appeared to be produced by infiltrating neutrophils 24–48 hr after infection, these cells were clearly not the source of IL-1β mRNA in afferent lymph. IL-1β specific mRNA appeared in afferent lymph before any substantial tissue damage or neutrophil influx had occurred and at a time when neutrophils were essentially absent from the lymph. Although migration of a cell population producing exclusively IL-1β would account for the lack of IL-1α mRNA in afferent lymph cells, a more likely explanation is that the delayed induction and prolonged expression of IL-1β mRNA, compared with IL-1α mRNA, that is observed *in vitro* in ovine alveolar macrophages[27] is observed in this situation *in vivo*. At this stage, the cellular origin of TNF-α, IL-1 and IL-8 mRNA in RNA extracted from afferent lymph cells remains obscure although this question is currently being addressed through cytokine-specific immunohistology and RT–PCR analysis of specifically selected cell populations. In addition, while afferent lymph cells expressing IL-8 mRNA may contribute to the IL-8 protein detected in afferent lymph, another source of this protein could be the IL-8-producing fibroblast or macrophage-like cells and infiltrating neutrophils within the inflammatory lesion that do not usually migrate through lymphatics.

The expression by afferent lymph cells of mRNA encoding cytokines other than those that are typically associated with inflammation was variable, as was the case with expression of these mRNA species in infected skin. In general, mRNA specific for IL-4, rather than IL-2 or IFN-γ was amplified from the RNA extracted from afferent lymph cells, although this was certainly not a consistent observation. There was also no obvious induction of these mRNA species in response to the infection, as mRNA coding for these cytokines were often detected prior to infection. Although it has not been possible to localize the IL-2, IL-4, IL-10 and IFN-γ mRNA detected in afferent lymph cells by *in situ* hybridization or to detect protein by immunohistology, the likely source is clearly the migrating CD4 and/or $\gamma\delta$ T cells. Lymphocytes trafficking through normal skin or through sites of acute inflammation have previously been shown to express a memory/activated phenotype.[9,10] In our model, infiltrating T cells in the skin and migrating T cells in the afferent lymph were predominantly positive for expression of IL-2Rα, which may be indicative of recent activation.[28] For $\gamma\delta$ T cells, this activation may have been the result of direct interaction with specific antigen within the lesion. $\gamma\delta$ T cells in other species have been demonstrated to respond to heat-shock and other stress-related proteins[29] and there is indirect evidence that ovine $\gamma\delta$ T cells may respond to stress-induced antigens.[15] Undoubtedly expression of these molecules would be upregulated in *L. cuprina*-induced inflammatory lesions. It seems unlikely, however, that activated, antigen-specific CD4 T cells would be recirculating back through an infection site within 24 hr of primary infection. The increased traffic of CD4 T cells and their contribution to the cytokine profile of afferent lymph may be dependent upon ongoing activation at remote sites by unrelated antigens. Infiltration would be a combined result of continued expression of adhesion molecule ligands on the T cells and the local synthesis of cytokines, such as RANTES, which are chemotactic for T cells.[30] In this particular case, the profile of T-cell cytokine expression in the inflammatory afferent lymph would be related to previous or ongoing antigenic experience, rather than any immediate response to the ectoparasite.

Irrespective of the cellular source or the initial stimulatory signal, both the pro-inflammatory and T-cell associated cytokines present in lymph draining the inflammatory lesion may influence the development of antigen-specific immunity in local lymphoid tissue. There is now a substantial body of data indicating that the presence of cytokines at the time of T-cell priming can profoundly influence the differentiation of the T-helper response and therefore the subsequent development and activation of immune effector mechanisms. While there has been extensive *in vivo* analysis of the effect of polarized T-helper responses in various models of infectious disease,[31] most analyses of the initiation of the polarization process have been carried out *in vitro*[32,33] and the cell populations that might represent the source of the differentiating cytokines remain obscure.[34] Our data demonstrate two mechanisms whereby localized acute inflammation may influence the development of immunity in draining lymphoid tissue. Firstly, cytokines such as IL-1, IL-8 and TNFα that mediate the inflammatory response can access draining lymphoid tissue via the afferent lymph. While a possible role for IL-8 in the development of antigen-specific immunity remains problematic, there is substantial *in vitro* and *in vivo* data on the potential impact of both IL-1 and TNF. Secondly, memory T cells and/or $\gamma\delta$ T cells recirculating through inflammatory sites and arriving at the draining lymphoid tissue may represent a source of cytokines that could influence the differentiation of a helper T-cell response following primary T-cell activation.

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