Immunology 1995 **86** 441–447

Identification of dendritic cells as a major source of interleukin-6 in draining lymph nodes following skin sensitization of mice

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SUMMARY

Skin sensitization with chemical allergens is associated with the activation and proliferation of T lymphocytes within lymph nodes draining the site of exposure. These events are accompanied by the secretion of interleukin-6 (IL-6) by lymph node cells (LNC). We have investigated the cellular source of IL-6 seventy-two hours following primary exposure of mice to the contact allergen oxazolone. Immunocytochemical analyses of sections of activated lymph nodes have revealed that cells expressing IL-6 are located within the T-dependent lymph node paracortex, with none present in lymphoid follicles. Cells which expressed IL-6 cofractionated exclusively with LNC of low buoyant density, the majority of which also expressed membrane Ia and had a dendritic morphology. Depletion of dendritic cells from LNC culture was associated with a significant decrease in the secretion of IL-6 by the residual population. These data demonstrate that dendritic cells are a major source of IL-6 within lymph nodes during primary immune responses to cutaneous antigens.

INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates various immune and inflammatory responses.[1] In addition to regulation of B-lymphocyte differentiation,[2] acute phase protein synthesis[3,4] and hematopoiesis,[5] IL-6 may be an important cofactor for T-lymphocyte activation.[3] This cytokine has been shown to act as a costimulus for T-lymphocyte activation and proliferation and can, in some experimental systems, replace the requirement for accessory cells.[6–8] We have demonstrated previously, using bioassay for IL-6, that the induction phase of contact sensitization is associated with the secretion by draining lymph node cells (LNC) of IL-6.[9] The magnitude of the IL-6 response correlated strongly with the vigour of LNC proliferation. However, complement depletion studies showed that the major source of IL-6 was not proliferating T lymphocytes.[9] Other cell populations that have been demonstrated to produce IL-6 include various accessory cells and B lymphocytes, both of which are known to reside in, or be recruited into, peripheral lymph nodes. Cultured Langerhans cells, which are phenotypically and functionally analogous to lymph node dendritic cells (DC), have recently been shown to express IL-6 mRNA and biologically active IL-6.[10] In the present study we have investigated the cellular source of IL-6 in lymph nodes 72 hr following primary exposure of mice to the contact allergen oxazolone. Immunocytochemical, and immunodepletion, studies have revealed that DC represent an important source of IL-6 during primary immune activation in peripheral lymph nodes.

Received 4 October 1994; revised 29 June 1995; accepted 4 July 1995.

Abbreviations: AEC, 3-amino-9-ethylcarbazole; AOO, acetone: olive oil (4:1); BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DC, dendritic cells; IL-6, interleukin-6; LNC, lymph node cells; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Animals

Young adult (8–12 weeks old) BALB/c mice (Harlan Olac, Bicester, Oxon, and Charles River Laboratories, Kent, UK) were used throughout these studies.

Chemicals and exposure

Oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one) was obtained from Sigma Chemical Co., St. Louis, MO, USA, and dissolved in vehicle (4:1 acetone: olive oil; AOO). Groups of mice received either 25 μl of 1% oxazolone dissolved in vehicle (n = 8–10) or an equal volume of vehicle alone (n = 10–40) on the dorsum of both ears, or were untreated (naive controls, n = 12).

Preparation of lymph node sections

Seventy-two hours following exposure, mice were killed and the draining auricular lymph nodes excised. Whole lymph nodes were snap frozen in isopentane (2-methylbutane, BDH Ltd,

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Poole, UK), which had been cooled in liquid nitrogen. Lymph nodes were embedded in OCT compound (Tissue Tek, Raymond A. Lamb, London), and 7 μm sections cut using a cryostat. Sections were transferred onto microscope slides, air dried and either used immediately or stored at −70° prior to analysis.

Preparation of lymph node cell suspensions and enrichment for DC

Single cell suspensions of LNC were prepared from lymph nodes isolated 72 hr following exposure to chemical or to vehicle alone, by mechanical disaggregation through sterile 200-mesh stainless steel gauze. Cells were washed and resuspended in RPMI-1640 culture medium (Gibco, Paisley, Renfrewshire, UK), supplemented with 25 mM HEPES, 400 μg/ml ampicillin, 400 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (RPMI–FCS). Viable cell counts were performed by exclusion of 0·5% trypan blue. For analysis of whole LNC suspensions, cells were adjusted to $2{\cdot}8 \times 10^5$ cells/ml in RPMI–FCS. Aliquots of 180 μl were loaded into cuvettes and centrifuged for 6 min (800 ***g***) in a Cytospin centrifuge (Shandon, Runcorn, Cheshire, UK).

Dendritic cells were enriched from unfractionated LNC populations by buoyant density centrifugation. The LNC concentration was adjusted to 5×10^6/ml and 8 ml aliquots were transferred to conical bottomed centrifuge tubes. Metrizamide (2 ml; 14·5% w/v analytical grade; Nycomed, Oslo, Norway) was layered gently under the LNC and centrifuged for 20 min (600 ***g***) at room temperature. Low-density cells accumulating at the interface were collected and washed once in RPMI–FCS. High-density cells, which formed a pellet following density centrifugation, were harvested as the DC-depleted (DC^-) population of LNC. DC were identified by direct morphological examination using interference contrast microscopy. Such cells are distinguished readily from small lymphocytes and display a characteristic 'ruffled' appearance with many surface extensions. DC enriched (DC^+) cell suspensions were adjusted to $1{\cdot}5 \times 10^5$/ml, and DC^- suspensions to $2{\cdot}8 \times 10^5$/ml, both in RPMI–FCS. Aliquots of 180 μl were loaded into cuvettes and centrifuged for 6 min (800 ***g***) in a Cytospin centrifuge. Cytospins were air dried and either used immediately or stored at −70° prior to analysis.

Immunocytochemistry

Frozen sections and cytocentrifuge preparations of LNC were air dried for 10–15 min at room temperature, followed by fixation in cold (4°) acetone for 5 min. The slides were then washed with phosphate-buffered saline (PBS; 3 × 5 min) and incubated with primary antibody, or an appropriate isotype-matched control antibody at an equivalent concentration (Table 1) for 30 min (or overnight incubation at 4° in the case of anti-IL-6 antibody). The specimens were washed in PBS (3 × 10 min) and incubated with biotinylated rabbit anti-rat IgG ($F(ab')_2$) (Serotec, Kidlington, UK), diluted to 5 μg/ml in 10% normal mouse serum, for 30 min. The slides were then washed as above (PBS; 3 × 10 min) and incubated with 50 μl Extravidin horseradish peroxidase complex or 50 μl Extravidin alkaline phosphatase complex (1/50 in PBS; Sigma) for 60 min and then washed as described previously. The substrates used were 3,3′-diaminobenzidine tetrahydrochloride with 3% (w/v) nickel chloride (DAB/NiCl) or 3-amino-9-ethylcarbazole (AEC) for horseradish peroxidase reactions, and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for alkaline phosphatase (BCIP/NBT; Sigma). Expression of IL-6 by LNC was detected using DAB/NiCl or BCIP/NBT, whereas AEC was utilized for the detection of other antigens. Following a 10 min incubation with substrate the slides were washed in PBS (10 min). Excess PBS was removed and the slides mounted in 9 : 1 glycerol : saline solution and sealed with nail varnish. Unless otherwise stated, all incubations were carried out at room temperature in a humid chamber. The slides were assessed microscopically. In LNC suspensions approximately 1000 cells were counted in random view fields. Results are expressed as the mean (± SD) percentage positive cells.

Table 1. Monoclonal antibodies used for immunocytochemistry

Antibody	Isotype	Concentration (μg/ml)
Rat anti-mouse IL-6 Genzyme	IgG1	1, 10*
Rat anti-mouse H2-IA Class II (Serotec; clone P7/7)	IgG2b	2
Rat anti-mouse Mac-1[11] (Serotec; clone M1/70)	IgG2b	1
Rat anti-mouse CD3 (Serotec; clone KT3)	IgG2a	1
Rat IgG1 myeloma protein (Serotec; clone IR27)	IgG1	1, 10*
Rat anti-human HLA class I (Serotec; clone YTH 862.2)	IgG2a	1
Rat anti-human HLA class I (Serotec; clone YTH 76.9)	IgG2b	2

* Lymph node sections: 1 μg/ml, cytospin preparations: 10 μg/ml.

Dendritic and T-cell depletion from draining LNC

LNC prepared as described above were incubated at 1×10^7 cells/ml at 37° for 30 min with 1 ml culture supernatant containing rat monoclonal (IgG2b) anti-DC antibody (33D1; a gift from Dr S. Knight, CRC Unit, Harrow, UK), or RPMI–FCS alone. Low toxicity rabbit complement was then added (10 ml, 1 : 12 dilution, Seralab, Crawley Down, UK) and cells were incubated for a further 45 min at 37° in a humidified atmosphere of 5% CO_2 in air. Cells were washed and resuspended in RPMI–FCS at 5×10^6 cells/ml. Production of IL-6 and lymphocyte proliferation were measured in parallel cultures of LNC.

For depletion of T lymphocytes, LNC were incubated at 2×10^7 cells/ml at 4° with 0·25 μg/ml rat monoclonal anti-Thy 1.2 antibody (Serotec, Kidlington, UK) or RPMI–FCS alone. LNC were washed twice and resuspended in RPMI–FCS supplemented with 10% low toxicity rabbit complement (Seralab, Crawley Down, UK) and incubated for a further 45 min at 37°. Cells were washed, layered onto Lymphosep (Seralab, Crawley Down, UK) and centrifuged at 500 ***g*** for 20 min. Cells accumulating at the interface were collected, washed and resuspended at 5×10^6 cells/ml.

Interleukin-6 production and cellular proliferation of draining LNC

In order to obtain supernatants for IL-6 analysis, LNC at

Table 2. Phenotypic characteristics of DC-enriched and -depleted populations

	Percentage positive cells*			
Antigen	Ox DC$^+$	Ox DC$^-$	AOO DC$^+$	AOO DC$^-$
IL-6	81 ± 3	0·7 ± 0·1	84 ± 8	0·6 ± 0·3
Ia	82 ± 3	35 ± 2	76 ± 2	38 ± 2
Mac-1 (strong)	0·43 ± 0·1	3 ± 0·5	0·52 ± 0·1	0·3 ± 0·3
Mac-1 (weak)	22 ± 4	—	23 ± 2	—
CD3	16 ± 4	62 ± 3	19 ± 4	60 ± 1

* Seventy-two hours following topical exposure to 1% oxazolone (Ox) or to vehicle alone (AOO), single cell suspensions of draining LNC were prepared and enriched for DC by density centrifugation. Cytospin preparations of DC-enriched (DC$^+$) and DC-depleted (DC$^-$) populations of LNC were analysed by immunocytochemistry for the expression of IL-6, Ia, Mac-1 and CD3. Approximately 1000 cells were counted in random fields and the mean positive cells (± SD) are presented.

5×10^6 cells/ml were cultured for 24 hr at 37° in a humidified atmosphere of 5% CO_2 in air. The concentration of IL-6 was determined using the B9 hybridoma cell line.[12] Each supernatant, or the standard preparation, was diluted serially with RPMI-1640 media supplemented with 5% heat inactivated FCS, 50 units/ml gentamicin and 5×10^{-5} M 2-mercaptoethanol, and incubated in microplates with B9 cells (5×10^4/ml) at 37° in a humidified atmosphere of 5% CO_2 in air. Cellular proliferation induced by IL-6 was assessed after 72 hr using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[13] Results are expressed as IL-6 units per ml (U/ml), relative to the international standard for human IL-6 (88/514, National Institute for Biological Standards and Control, South Mimms, UK, 1 U ≈ 1 pg).

Proliferation was assessed in parallel cultures of LNC which were cultured for 24 hr at 37°in a humidified atmosphere of 5% CO_2 in air, in the presence of 2 μCi of ^{3}H-methyl thymidine (^{3}HTdR, specific activity 2·5 Ci/mmol; Amersham International, Amersham, UK). Culture was terminated by automated harvesting onto glass fibre filters, and thymidine incorporation determined by β scintillation counting. Results are expressed as mean (± SD) counts per minute (c.p.m.).

Statistical analysis

Statistical analyses were performed using a paired one-tailed Student's *t*-test with 95% confidence interval. *P* values of less than 0·05 were considered significant.

RESULTS

Detection of cells expressing IL-6 in lymph node sections

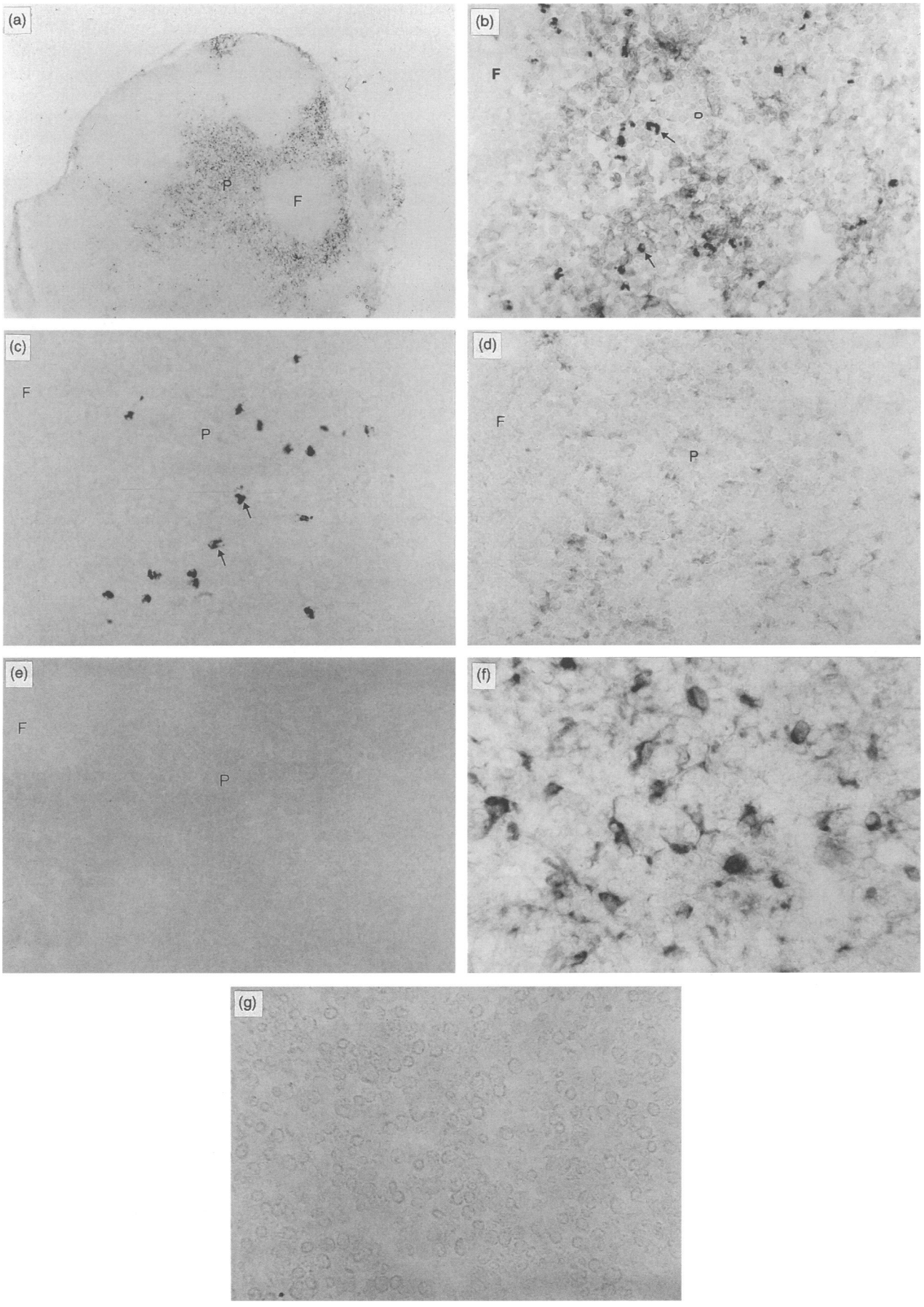
Immunocytochemical analyses of IL-6 expression in lymph nodes prepared 72 hr following topical exposure to 1% oxazolone revealed that a network of IL-6$^+$ cells was located throughout the T cell-dependent lymph node paracortex, with none present in lymphoid follicles (Fig. 1a,b). The use of a horseradish peroxidase detection system also resulted in the appearance of cells expressing endogenous peroxidase activity, possibly macrophages. These were observed as intensely stained cells with both the specific antibody and with the isotype-matched control and were readily distinguishable from IL-6$^+$ cells (Fig. 1b,c). Cells expressing IL-6 were observed throughout the paracortical regions of lymph nodes prepared from mice exposed to vehicle alone, although the staining was much less intense and few endogenous peroxidase-positive cells were identified (Fig. 1d). Expression of IL-6 within lymph nodes prepared from naive mice did not differ from that observed within lymph nodes from mice treated with vehicle alone (data not presented). No staining was observed with the isotype-matched control antibody (Fig. 1c,e). Since the endogenous peroxidase activity observed could not be quenched with hydrogen peroxide, some analyses were performed using an alkaline phosphatase detection system. Using this approach, a similar distribution of cells expressing IL-6 was observed throughout the paracortex and at high magnification these cells appeared dendritic in nature (Fig. 1f). No staining was observed with the isotype control antibody (Fig. 1g).

Identification of cells expressing IL-6 in LNC suspensions

Seventy-two hours following topical exposure to 1% oxazolone or vehicle alone, single cell suspensions of LNC were prepared. Cytospin preparations of LNC were analysed by immunocytochemistry. Analysis of unfractionated LNC revealed that IL-6$^+$ cells comprised a small percentage of the population (approximately 2% in naive populations or after exposure to vehicle alone; 5% following treatment with oxazolone), and were large, irregularly shaped cells (Fig. 2a). DC$^+$ and DC$^-$ populations of LNC were prepared and were analysed in serial cytospin preparations for the expression of IL-6 and the membrane determinants Ia, CD3 and Mac-1.

Approximately 80% of cells in the low buoyant density (DC$^+$) fraction of LNC expressed IL-6 (Fig. 2b, Table 2), although the intensity of staining varied between cells. No positive staining was observed with the isotype-matched control antibody (Fig. 2c). In serial cytospins a similar number of cells was Ia$^+$ (Table 2). Although the low buoyant density cells isolated from mice treated with vehicle were also IL-6$^+$ (Table 2), oxazolone-activated lymph nodes contained significantly higher numbers of DC per node than did nodes isolated following treatment with vehicle alone (26 290 ± 1284 following exposure to oxazolone; 3348 ± 395 after treatment with vehicle [mean ± SEM of four experiments]). Thus, lymph nodes isolated 72 hr following topical application of oxazolone may contain significantly more IL-6$^+$ cells than lymph nodes isolated following treatment with vehicle alone. Approximately 25% of low buoyant density cells expressed very low levels of Mac-1 (Table 2), as has been reported previously.[14,15] A minority population (approximately 0·5%) of cells in the low buoyant density fraction expressed higher levels of Mac-1. The DC$^-$ population obtained following exposure to oxazolone contained approximately ten times the number of strongly Mac-1$^+$ cells (3%) than the DC$^-$ population isolated following treatment with vehicle alone (0·3%).

Large T lymphoblasts may cofractionate with DC on metrizamide gradients,[16] and in these experiments up to 20% of cells within the DC$^+$ fraction were found to express CD3. Following topical exposure to oxazolone or to vehicle alone,



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the DC^- population of LNC was comprised of 60–62% $CD3^+$ T lymphocytes and 35–38% Ia^+ B lymphocytes.

Depletion of DC reduces IL-6 production by draining LNC

Following topical exposure to 1% oxazolone, draining LNC suspensions were incubated with 33D1 anti-DC antibody, or RPMI–FCS alone, plus complement. Production of IL-6 and [^{3}H]TdR incorporation was assessed in parallel. Following incubation of LNC with RPMI–FCS and complement, IL-6 production and [^{3}H]TdR incorporation did not differ significantly from values obtained for control cells incubated in medium alone and were similar to results obtained previously[9] (Table 3). However, incubation of LNC with 33D1 and complement reduced IL-6 secretion by more than 75% ($P \leq 0{\cdot}05$; Table 3), but had no significant effect on [^{3}H]TdR incorporation. In contrast, depletion of T cells from culture was associated with a marked reduction in proliferation and an increase in the secretion of IL-6, as reported previously.

DISCUSSION

The induction phase of contact sensitization is characterized by, and dependent upon, the activation and proliferation of lymphocytes within lymph nodes draining the site of exposure.[17–19] Activation of T lymphocytes, in addition to antigen presentation and recognition through the MHC–antigen–T-cell receptor complex, is considered to require a number of accessory cell-derived signals. The initial binding event between antigen-presenting cells and T lymphocytes requires the interaction between cell surface adhesion molecules including intercellular adhesion molecule-1 and lymphocyte function antigen-1. The interactions of other cell surface molecules such as those between CD28, CTLA-4 and their respective B7 cell ligands may also be important[20,21] in delivering costimulatory signals to the lymphocyte.

Cytokines are also considered important for the initial costimulatory events which lead to T-lymphocyte activation. IL-6 has been found to replace the requirement for antigen-presenting cells in models of T-lymphocyte activation[6,8] both *in vitro* and *in vivo*,[4,22] and may therefore provide costimulatory signals for the activation of T lymphocytes. We have demonstrated previously that 72 hr following the initiation of contact sensitization, cells within lymph nodes were stimulated to produce IL-6, and that this correlated closely with the proliferative activity of LNC.[9]

Cell populations which have been shown to produce IL-6 include T and B lymphocytes and macrophages,[1] each of which are present in peripheral lymph nodes. Cultured Langerhans cells have also been shown recently to express mRNA for IL-6 and to secrete biologically active protein.[10] In the experiments described here, cells expressing IL-6 were located in the T-dependent lymph node paracortex. Lymphoid follicles were not associated with IL-6 expression, indicating that B lymphocytes are not a significant source of this cytokine in lymph nodes during primary responses to chemical allergens.

Although cells were initially analysed for the coexpression of IL-6 and cell surface markers by double immunocytochemistry, this method proved unreliable and difficult to interpret. Therefore, to identify further the cell population responsible for IL-6 production the distribution of cells which expressed IL-6 in isolated cell populations was compared with that of cell surface molecules characteristic of T lymphocytes, macrophages and DC. Although a number of monoclonal antibodies have been described as useful for the identification of DC,[23–25] there is evidence that these may not recognize all DC in immunocytochemical procedures.[26] However, DC may be distinguished on the basis of their low buoyant density and expression of markers such as membrane Ia, which may be particularly useful for the identification of DC in isolated cell populations.

IL-6 expression cofractionated with LNC of low buoyant density. In serial cytospins the majority of low buoyant density cells expressed membrane Ia and displayed the extended membrane processes characteristic of DC (not shown). Complement depletion of LNC with an antibody specific for DC was associated with a decrease of greater than 75% in the secretion of IL-6 by the residual LNC population, indicating that DC represent the major cellular source of IL-6 within the draining lymph node. Depletion of T lymphocytes from LNC culture enriched for IL-6-producing cells, confirming our previous observation[9] that T lymphocytes were not a major cellular source of IL-6. The distribution and frequency of Mac-1^+ and IL-6^+ cells within cell suspensions indicate that the Mac-1^+ cells are unlikely to be the major source of IL-6 in draining lymph nodes following contact sensitization. Lymph nodes activated by oxazolone contained significantly more Mac-1^+ cells and endogenous peroxidase than did lymph nodes removed from mice treated with vehicle alone, indicating that Mac-1^+ cells, including macrophages, may account for the endogenous peroxidase activity observed. These results indicated also that topical exposure to chemical allergen may be associated with an influx of macrophages into the lymph node paracortex.

Dendritic cells are considered to be the principal cells responsible for presenting antigen during the primary immune response, both *in vitro* and *in vivo*.[13,24] However, although the primary activation of T lymphocytes requires both antigen and additional signals derived from accessory cells, only thymic and splenic DC have been shown to produce IL-6.[27–29] Helle and co-workers found that low-density thymic cells produced IL-6[27] and their observations were confirmed by Papiernik and co-workers,[28] who demonstrated that transiently adherent, Ia-positive, thymic and splenic dendritic cells expressed IL-6 when

Figure 1. Detection of cells expressing IL-6 in lymph node sections. Seventy-two hours following topical exposure to 1% oxazolone or to vehicle alone lymph node sections were prepared. Lymph node sections prepared following exposure to oxazolone were analysed for the expression of IL-6 (a, magnification ×16·8; b, magnification ×105) and isotype control antibody staining (IgG1; c, ×105). Lymph nodes prepared following topical exposure to vehicle alone were also analysed for the expression of IL-6 (d, ×105) and isotype control antibody staining (e, ×105). Non-specific endogenous peroxidase$^+$ cells are indicated with an arrow. The expression of IL-6 (f, ×168) and negative isotype control antibody staining (g, ×168) in oxazolone-activated lymph nodes were also analysed using an alkaline phosphatase endpoint technique (substrate BCIP/NBT). F = lymph node follicle; P = lymph node paracortex.

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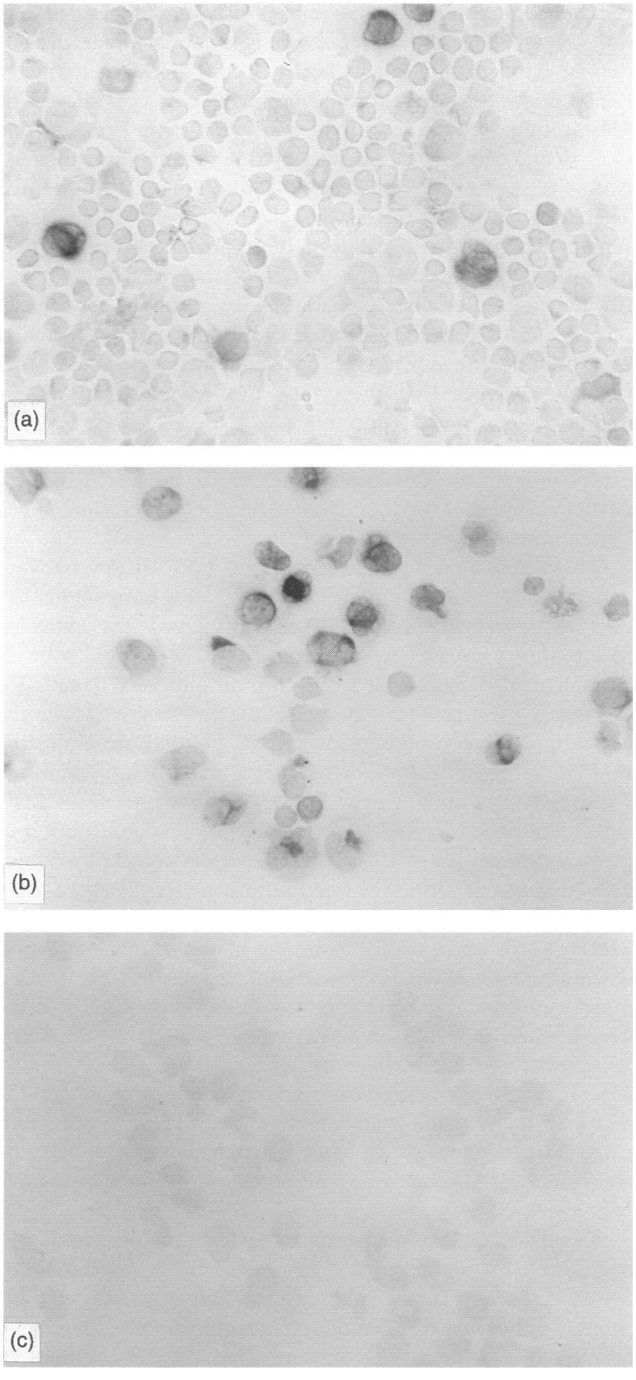

Figure 2. Identity of IL-6-expressing cells in lymph node cell suspensions. Seventy-two hours following topical exposure to 1% oxazolone, lymph node cell suspensions were prepared and analysed for the expression of IL-6 by immunocytochemistry (a). One representative experiment of four is illustrated. Dendritic cell-enriched and -depleted cell populations were prepared by density centrifugation. DC^+ were analysed for the expression of IL-6 (b) and isotype control staining (IgG1; c). In each case IL-6 was detected using a horseradish peroxidase detection system with the substrate DAB/NiCl. Magnification ×168. One representative experiment of four is shown.

Table 3. IL-6 production and proliferation following depletion of DC and T cells from LNC

Treatment	IL-6 U/ml	[^{3}H]TdR incorporation (c.p.m. × 10^{-3})
RPMI–FCS alone	237·1 ± 17·2	59·9 ± 7·9
DC-depleted	67·3 ± 2·6*	53·6 ± 0·8
Control	194·6 ± 36·7	85·3 ± 4·9
T-cell-depleted	688·9 ± 47·8	9·0 ± 0·5

Seventy-two hours following topical exposure of mice to 1% oxazolone, draining LNC were prepared. Cells were incubated with RPMI–FCS alone, RPMI–FCS and complement (control), or rat anti-DC monoclonal antibody (33D1) and complement (DC-depleted). T cells were depleted as described previously.[9] Viable cells were cultured for 24 hr and the production of IL-6 and [^{3}H]TdR incorporation was assessed.[9]

* Significantly reduced compared to control ($P \leq 0{\cdot}05$).

cultured for 24 hr *in vitro*. In addition, splenic cell lines with some characteristics of DC, including the expression of Ia and low level stimulation of T-lymphocyte proliferation, have been shown to produce IL-6.[29] The data presented here suggest that DC are the major source of IL-6 during primary immune responses in peripheral lymph nodes. We have demonstrated that following topical exposure to chemical allergen, cells within the lymph node paracortex expressed increased levels of IL-6, as compared with naive and vehicle-treated mice. These cells express the characteristics of DC. The number of $IL\text{-}6^+$ DC increased significantly following topical exposure to oxazolone, this may account for the increased IL-6 production by LNC following allergen exposure.[9] These results show that lymph node DC have the potential to secrete IL-6 and represent the major cellular source of this cytokine in allergen-activated lymph nodes. The production of IL-6 by lymph node DC may be an important element in the activation of T lymphocytes and the induction of cutaneous immune responses.

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