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The kinetics of cytokine production by draining lymph node cells following primary exposure of mice to chemical allergens

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SUMMARY

Skin sensitization with chemical allergens is associated with the activation and proliferation of lymphocytes in lymph nodes draining the site of exposure. As lymphocyte activation is regulated by the action of cytokines, we have investigated the nature and kinetics of cytokine production by draining lymph node cells (LNC) from mice, following their primary exposure to chemical allergens. Both interleukin-1 (IL-1) and IL-6 were induced in a biphasic manner following primary exposure of mice to oxazolone or to dicyclohexylmethane-4,4′-diisocyanate (HMDI). The initial phase of production occurred when LNC were prepared from mice 8–20 hr following exposure, while the second peak was coincident with the maximal proliferative response at 72 hr. Increased IL-4 production was observed only when LNC were prepared 96 hr following sensitization. Despite vigorous lymphocyte proliferation there was no evidence for IL-2 production by draining LNC. The ordered and transient pattern of cytokine production that occurs during the afferent phase of contact sensitization suggests that sequential cytokine signals may be involved in regulating the characteristics of the response generated within the draining lymph node.

INTRODUCTION

Following primary epidermal exposure to contact allergens there is extensive proliferation of antigen-specific and non-specific T lymphocytes within draining lymph nodes. Previous studies have shown that this response is associated with the production, by draining lymph node cells (LNC), of cytokines such as interleukin-1 (IL-1), IL-2, IL-3, IL-4 and IL-6, tumour necrosis factor-α and interferon-γ.[1–6]

T-lymphocyte activation is considered to be at least a two-step process, requiring both antigen presentation and costimulatory signals. The exact nature of these additional signals is unclear, although there is evidence that both cell membrane interactions and soluble cytokines are important.[7–9] Putative cytokine signals include IL-1 and IL-6, which have been shown to act, either alone[10–12] or in concert,[13] as costimuli for T-lymphocyte activation in some experimental systems. Both cytokines are required at early stages of T-lymphocyte activation and may control progression through the cell cycle.[14] Activated T lymphocytes are found usually to produce IL-2 and display up-regulated IL-2 receptor (IL-2R) expression.[15] Interaction of IL-2 with its receptor is considered to be an important step in the stimulation of T-cell proliferation and clonal expansion. In addition to IL-2, IL-4 can provide stimulatory signals for T-lymphocyte proliferation and is the autocrine growth factor for T-helper type 2 (Th2) cells.[16]

In a previous study we demonstrated that draining LNC were stimulated to produce IL-1 within hours of primary exposure of mice to the contact allergen oxazolone.[1] In the present investigation we examined the extended kinetics of induction of IL-1, and other cytokines considered to be important for T-lymphocyte activation and proliferation, by dicyclohexylmethane-4,4′-diisocyanate (HMDI), a chemical shown previously to cause contact sensitization of mice.[17] We confirmed our observations with oxazolone and report that both chemicals induce, with similar and ordered kinetics, the production of IL-1, IL-4 and IL-6 by draining LNC, in the absence of detectable IL-2.

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Abbreviations: AOO, acetone : olive oil (4 : 1); DC, dendritic cell; HMDI, dicyclohexylmethane-4,4′-diisocyanate; IL, interleukin; LC, Langerhans' cell; LNC, lymph node cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Th, T-helper lymphocyte.

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MATERIALS AND METHODS

Animals

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

Chemicals

HMDI was purchased from Aldrich Chemical Co. Ltd (Gillingham, UK). Oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one) was obtained from Sigma Chemical Co. (St Louis, MO). Both chemicals were dissolved in 4 : 1 acetone : olive oil (AOO).

Exposure to chemicals and preparation of LNC

Groups of mice received either 25 μl of 1% test chemical dissolved in vehicle on the dorsum of both ears (n = 3–6), or were unexposed (naive controls, n = 10–15). AOO has been shown previously to elicit neither significant IL-6 production nor significant levels of LNC proliferation.[4] At various times following exposure, mice were killed and the draining (auricular) lymph nodes were excised and pooled for each experimental group. Single-cell suspensions of LNC were prepared by mechanical disaggregation through sterile 200-mesh stainless steel gauze. Cells were washed and resuspended in RPMI-1640 culture medium (Gibco, Paisley, UK), supplemented with 25 mM HEPES, 400 μg/ml ampicillin, 400 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS). Viable cell counts were performed by exclusion of 0·5% trypan blue.

Cytokine production and cellular proliferation by draining LNC

To obtain supernatants for cytokine analysis, LNC (5×10^6 cells/ml) were seeded into 24-well tissue culture plates and cultured in replicate wells for 24 hr at 37° in a humidified atmosphere of 5% CO_2 in air. Culture was terminated by centrifugation. Replicate supernatants were pooled and stored at −70° prior to analysis.

The concentration of cytokines in supernatants from LNC was determined using cytokine-specific bioassays. IL-1 was assayed using the D10(N4)M subline of the D10.G4.1 T-cell clone.[18] The B9 hybridoma cell line was used to measure IL-6.[19] Concentrations of IL-2 and IL-4 were determined using the T-cell lines CTLL-2[20] and F4.4K.6,[21] respectively. Briefly, each supernatant or laboratory standard preparation was diluted serially with RPMI-1640 supplemented with 50 U/ml of gentamicin and 5×10^{-5} M 2-mercaptoethanol plus 5% heat-inactivated FCS for the IL-6 assay and 10% FCS for IL-1, IL-2 and IL-4 assays. Indicator cells at 10×10^4/ml or 5×10^4/ml (IL-6 assay), were added and incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Cellular proliferation induced by cytokines was assessed after 72 hr using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colourimetric method.[22] Results are expressed relative to the interim standard for human IL-1β [86/552 National Institute for Biological Standards and Control (NIBSC), South Mimms, UK: 1 U $\approx$ 10 pg], the international standard for human IL-2 (86/504 NIBSC: 1 U $\approx$ 200 pg), recombinant murine IL-4 (Genzyme Diagnostics, Kent, UK: 1 U $\approx$ 100 pg), and a recombinant human IL-6 preparation[23] provided by Dr L. A. Aarden (Central Laboratory of the Netherland Red Cross Blood Transfusion Service, Amsterdam, the Netherlands: 1 U $\approx$ 1 pg. Although both F4 and CTLL-2 cell lines can respond to both IL-2 and IL-4, experience in this laboratory indicates that CTLL-2 responds only to concentrations of IL-4 above 10 U/ml, and F4 only to >2 U/ml IL-2. In contrast, CTLL-2 cells respond to 0·6 U/ml IL-2 and F4 to >0·2 U/ml IL-4.

Proliferation was assessed in parallel cultures of LNC (5×10^6 cells/ml), 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}H[TdR]; 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.

Statistical analysis

Results are expressed as mean [^{3}H]TdR incorporation in replicate LNC cultures, and as the mean of replicate determinations of cytokine concentration in parallel pooled supernatants. For clarity means ± standard deviation (SD) are shown only for cytokine concentrations: SD of [^{3}H]TdR values were found consistently to be less than 10% of the mean. Statistical analyses were performed using a paired Student's t-test. P-values less than 0·05 were considered to be significant.

RESULTS

IL-1 and IL-6 production by draining LNC is biphasic

To investigate the kinetics of cytokine production by draining LNC, independent kinetic experiments were performed with cells prepared from mice exposed to HMDI, either 8 to 24 hr or 48 to 120 hr, previously. In each of four experiments IL-1 concentrations in supernatants from cultured draining LNC were increased significantly ($P \leqslant 0{\cdot}01$), compared to naive controls, when cells were prepared at 8–20 hr post-sensitization. In three of these experiments, peak IL-1 concentrations were observed when LNC were prepared at 12 hr (representative experiment, Fig. 1a). In the fourth experiment the greatest IL-1 concentration was produced by cells taken at 20 hr (data not shown). By 24 hr, IL-1 concentrations had decreased to values comparable with those obtained for control mice. No significant increase in [^{3}H]TdR incorporation was recorded at any period prior to 24 hr. In each of two separate experiments where cells were taken from mice at 48–120 hr post-sensitization, IL-1 concentrations were significantly elevated at 48, 72 and 96 hr ($P \leqslant 0{\cdot}01$), with the peak at 72 hr (representative experiment, Fig. 1b). Although some interexperimental variation in the absolute IL-1 concentrations was recorded, the biphasic pattern of induction of IL-1 was consistent in all experiments performed.

The kinetics of IL-6 production in these experiments was similar to those observed for IL-1. Peak production was detected when LNC were prepared at 12 hr in three experiments (representative experiment, Fig. 2a), and at 20 hr in another experiment. The second peak of IL-6 production coincided with peak [^{3}H]TdR incorporation and IL-1 production at 72 hr (representative experiment, Fig. 2b). By 120 hr IL-1 and IL-6 production and [^{3}H]TdR incorporation had decreased to less than 25% of maximum values and was approaching that of LNC prepared from control mice. To determine whether the biphasic response pattern of cytokine induction was unique to HMDI, mice were sensitized with 1% oxazolone and the draining lymph nodes removed over a 96-hr or a 120-hr period. Both IL-1 (Fig. 3a) and IL-6 (Fig. 3b) were elevated significantly ($P \leqslant 0{\cdot}01$), when cells were prepared 8 hr post-sensitization, and were decreased again when cells were taken at 12 hr. A second peak of IL-1 and IL-6 production was observed in supernatants from cells prepared at 72 hr. Maximal incorporation of [^{3}H]TdR was again coincident with the second peak of IL-1 and IL-6 production (Fig. 3). Although quantitative differences in cytokine production were recorded, the biphasic pattern of induction of both IL-1 and IL-6 was maintained in all experiments.

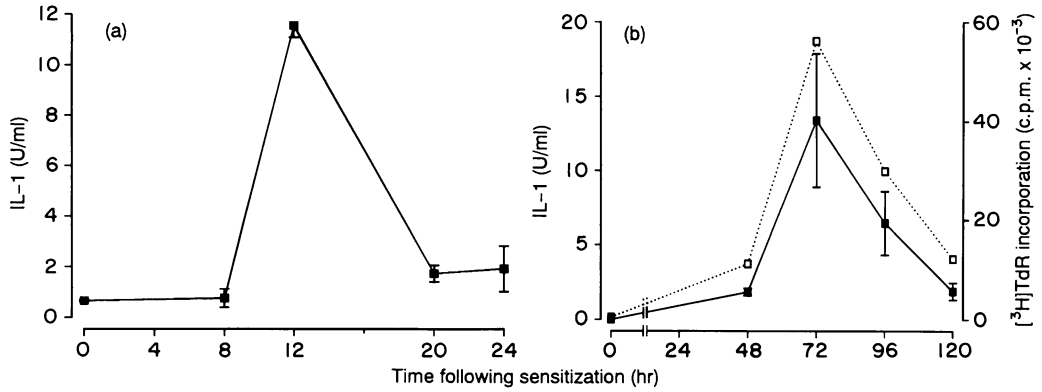

Figure 1. Kinetics of IL-1 production and proliferation by LNC from mice sensitized with HMDI. Following topical application of 1% HMDI draining LNC were collected at 8–24 hr (a) or 48–120 hr (b), and cultured for 24 hr. The concentration of IL-1 in supernatants (■ a and b) and [^{3}H]TdR incorporation (□ b) in parallel cultures were determined. One representative experiment is shown. Time 0 represents naive (unexposed) mice.

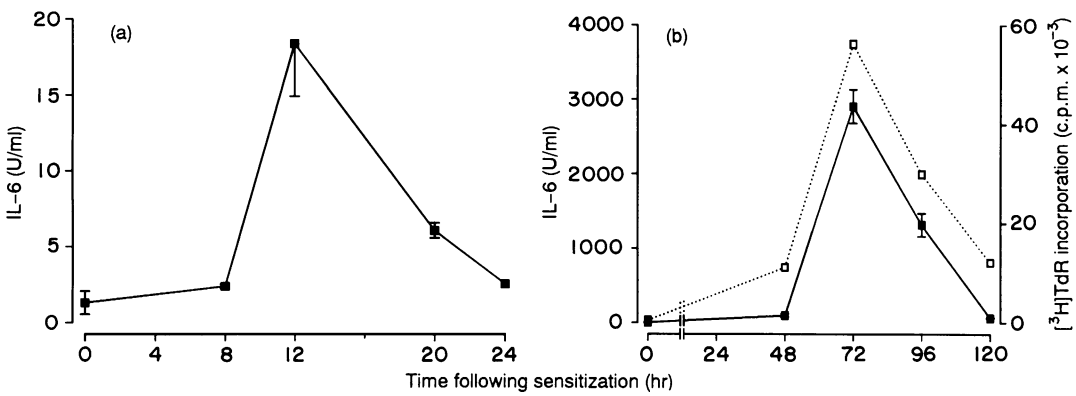

Figure 2. Kinetics of IL-6 production and proliferation of LNC from mice sensitized with HMDI. Following topical application of 1% HMDI draining LNC were collected at 8–24 hr (a) or 48–120 hr (b), and cultured for 24 hr. The concentration of IL-6 in supernatants (■ a and b) and [^{3}H]TdR incorporation in parallel cultures (□ b) were determined. One representative experiment is shown. Time 0 represents naive mice.

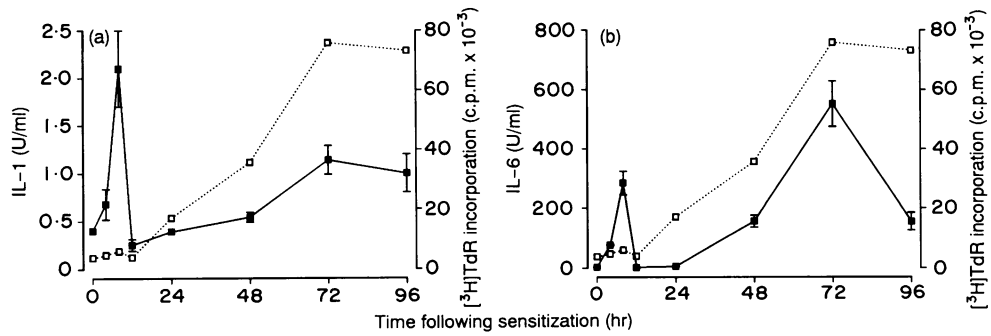

Figure 3. Kinetics of IL-1 and IL-6 production and proliferation of LNC from mice sensitized with oxazolone. Following topical exposure to 1% oxazolone draining LNC were prepared at the times indicated and cultured for 24 hr. Concentrations of IL-1 (a) and IL-6 (b) and [^{3}H]TdR incorporation were determined. One experiment is shown. Time 0 represents naive mice.

IL-4, and absence of IL-2, production by draining LNC

IL-2 and IL-4 concentrations were determined in the supernatants described above. No IL-2 was detected at any time-point in each of six independent experiments where mice were sensitized to HMDI or in each of two experiments with oxazolone (limit of detection = 0·6 U/ml). The IL-4 concentration in supernatants from cells collected up to 72 hr following sensitization was not increased above that detected in supernatants from cells prepared from control animals, but was increased significantly in supernatants from cells prepared 96 hr following sensitization with HMDI ($P \leqslant 0{\cdot}01$) (representative experiment, Fig. 4a). By 120 hr IL-4 concentrations had

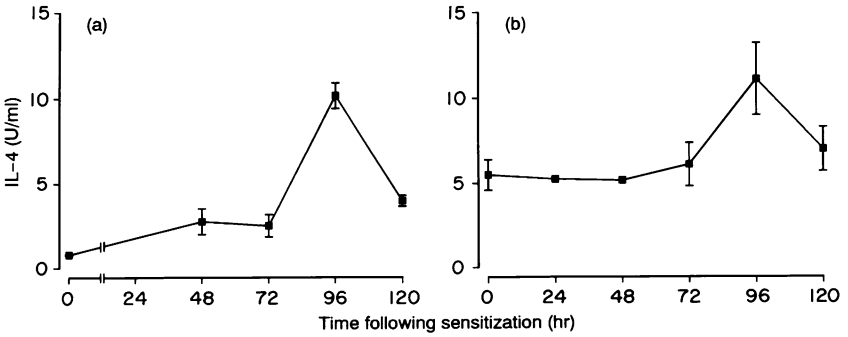

Figure 4. Kinetics of IL-4 production by LNC from mice sensitized to HMDI or oxazolone. Following topical application of 1% HMDI (a) or 1% oxazolone (b), draining LNC were prepared at the times indicated and cultured for 24 hr, prior to determination of IL-4 concentration in supernatants. One representative experiment is shown. Time 0 represents naive mice.

decreased to values similar to those obtained with unsensitized controls. The production of IL-4 by cells prepared at 96 hr post-sensitization was confirmed in experiments where mice were sensitized with oxazolone (Fig. 4b).

DISCUSSION

Following primary exposure to contact sensitizing chemicals, the allergen associates with epidermal Langerhans' cells (LC) and is transported via afferent lymphatics to lymph nodes draining the site of application.[24] Presentation of antigen to specific T lymphocytes is followed by lymphocyte proliferation within the draining lymph node. Although antigen alone is insufficient to induce the activation of T lymphocytes, the exact nature of the accessory signals required is unclear. The interaction of a variety of membrane-bound ligands with their cellular receptors may be important,[9] but there is evidence that cytokines such as IL-1 and IL-6 may also provide secondary signals, as these molecules have been found to be able to replace the requirement for accessory cells in some experimental systems.[10–13] Together with antigen, these signals promote entry of T lymphocytes into the G_1 phase of the cell cycle. The subsequent progression through the cell cycle and proliferation is considered to be dependent upon the action of T-cell growth factors such as IL-2 and/or IL-4.[16]

Previous studies have demonstrated that cells within draining lymph nodes can produce a number of cytokines, including IL-1, IL-2, IL-4 and IL-6, following the primary application of contact sensitizing chemicals.[1,2,4,6] Here, we demonstrate that, in the absence of subsequent antigenic stimulation *in vitro*, these cells produce IL-1 and IL-6 in a biphasic manner; an early peak at 8–20 hr post-sensitization was followed by a second peak at 72 hr following sensitization, the second peak of production being coincident with maximum [^{3}H]TdR incorporation. The early production of IL-1 and IL-6 is consistent with evidence of a role for these cytokines during the initial stages of lymphocyte activation. The capacity of draining LNC to produce IL-1 and IL-6 appears within hours of skin sensitization and the timing of this production is similar to that of the initial influx of antigen-bearing dendritic cells (DC) into regional lymph nodes.[24] This accumulation of DC has been shown previously to correlate with the vigour of subsequent LNC proliferation.[25] DC accumulating in the draining lymph nodes of contact sensitized mice derive from epidermal LC,[26] which undergo a phenotypic and functional maturation while in transit from skin to draining lymph nodes.[27,28] Cultured LC undergo similar changes *in vitro*, display increased expression of membrane major histocompatibility complex (MHC) class II determinants, augmented antigen-presenting activity,[29] and have been shown recently to produce IL-6.[30] As culture of LC induces similar changes to those occurring *in vivo*, it is likely that the DC which accumulate in lymph nodes may represent a source of IL-6 following contact sensitization. Epidermal LC are reported also to produce IL-1β, with mRNA for this cytokine being up-regulated within 15 min of topical exposure of mice to a range of contact allergens.[31,32] Studies performed previously in this laboratory indicated that the initial peak of IL-1 produced by draining LNC following contact sensitization was IL-1β.[1] It is possible therefore, that DC accumulating in draining lymph nodes may represent a source of both IL-1 and IL-6 following contact sensitization. The co-ordinate early expression of IL-1 and IL-6 may reflect evidence indicating a requirement for the simultaneous presence of these two cytokines prior to T-lymphocyte activation and proliferation.[33]

IL-1 and IL-6 production was again increased in supernatants from cells prepared 72 hr post-sensitization, coincident with the peak LNC proliferative response. Given the temporal sequence of events it is unlikely that this second peak of production of IL-1 and/or IL-6 is responsible for the simultaneous vigorous LNC proliferation observed. We believed that the production of IL-6 72 hr post-sensitization might be a result of, rather than a signal for, lymph node activation. However, recent evidence demonstrated that T lymphocytes were not the major source of IL-6 production in the second peak, as T-lymphocyte depletion largely abolished proliferation but increased IL-6 production.[4] The role of IL-1 at this time is unclear also, although IL-1β does reduce the flow of fluid and cells through efferent lymphatic vessels,[34] and may therefore contribute to the retention of lymphocytes within lymph nodes, a characteristic feature of contact allergic responses.

The significance of the differences in concentrations of IL-1 and IL-6 produced at different times and with the different chemicals is unclear. Despite the consistency of the biphasic nature of production there was variation in the absolute level of IL-1 produced, as has previously been described.[1] Quantitative differences in the IL-6 responses induced were also recorded. Although both HMDI and

oxazolone induced IL-6 production in a biphasic manner, topical exposure to oxazolone induced a more rapid and vigorous early peak of IL-6 production than did HMDI. Such differences in IL-6 production may reflect differences in the kinetics and number of DC accumulating in lymph nodes draining the site of topical exposure.[23]

Proliferation of T lymphocytes is associated classically with the production of IL-2 and its subsequent interaction with the IL-2R.[15] However, although there was a vigorous LNC proliferative response following topical exposure to both HMDI and oxazolone, there was no evidence to suggest that this was accompanied by the production of IL-2. The failure to detect IL-2 bioactivity could theoretically reflect consumption of this cytokine by activated T lymphocytes, as addition of anti-IL-2R α chain antibody to lymphocyte cultures has been reported to allow increased recovery of IL-2.[35] However, the total inability to detect IL-2 suggests that the concentrations must in any case be very low. An alternative explanation could be coproduction of a factor preventing effective detection of IL-2. Indeed we have shown that these supernatants inhibited the response to 4 U/ml IL-2 by 20–30%, at the highest concentration of supernatant assayed (J. C. Hope, R. J. Dearman, I. Kimber & S. J. Hopkins, unpublished observations). Other studies, in which mice were exposed topically to the contact allergens picryl chloride[3] or oxazolone,[6] support our observations. Neither IL-2 messenger RNA (mRNA), nor IL-2 protein production could be detected in the absence of additional *in vitro* stimulation. However, another group has reported the induction of IL-2 by LNC derived from mice sensitized to picryl chloride.[2] IL-2 mRNA production was detectable by reverse transcriptase polymerase chain reaction at 1 and 2 days following sensitization. Maximal IL-2 activity was detected in supernatants of day 3 cells, when IL-2 mRNA was at background levels. It is notable that the reported IL-2 activity peak coincided with, rather than preceded, the peak proliferative response, like the second peak of IL-1 and IL-6 in the present study. The weight of evidence therefore suggests that the primary proliferative response *in vivo* may be largely IL-2 independent, as has been demonstrated recently for the *in vitro* response to concanavalin A.[36]

IL-4 can also act as a growth factor for antigen-specific T lymphocytes, particularly in synergy with IL-1. Others have demonstrated the presence of IL-4 mRNA transcripts in draining LNC 3–4 days post-sensitization with picryl chloride, although no IL-4 bioactivity could be detected.[2] As IL-1 has been shown to be a prerequisite for IL-4 production by Th2 cells,[16,37] the elevation in IL-1 observed at 8–20 hr and at 3 days could promote the subsequent production of IL-4. The kinetics of IL-4 production and LNC proliferation suggest that IL-4 cannot be responsible for inducing the proliferative response. In contrast, IL-4 may be involved in the subsequent down-regulation of cellular activation within the lymph node, as it has been implicated in the inhibition of both cytokine production by monocytes[38] and the down-regulation of B-lymphocyte proliferation.[39] Alternatively administration of anti-IL-4 antibodies during antigen priming has demonstrated that IL-4 plays an important role in establishing the lymphokine-producing T-cell phenotype and determines whether subsequent responses will involve IL-4.[40]

The role and the cellular sources of IL-1, IL-4, IL-6 and other cytokines in draining lymph nodes remain unclear at present. However, the regulated pattern of induction of these cytokines indicates that they may play an important part in controlling the lymph node response following primary contact sensitization. This primary response is in turn likely to be crucial to the nature of any secondary response.

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