Distinct In Vivo and In Vitro Cytokine Profiles of Draining Lymph Node Cells in Acute and Chronic Phases of Contact Hypersensitivity: Importance of a Type 2 Cytokine-Rich Cutaneous Milieu for the Development of an Early-Type Response in the Chronic Phase

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Contact hypersensitivity (CH) is often regarded as in vivo manifestations of type 1 cytokine-mediated immunity to epicutaneously applied haptens (1). In CH, Ag-specific effector T cells that mediate CH responses are postulated to extravasate at the site of Ag challenge, and upon recognition of Ag/MHC complexes on local APC they release type 1 cytokines that, in turn, recruit and activate variety of nonspecific inflammatory cells. Regional draining lymph nodes (LN) are believed to be rich sources of these Ag-specific effector type 1 cells, because the literature still reflects a widespread acceptance of the concept that the clonal expansion and differentiation into effector T cells take place in these secondary lymphoid organs, and upon subsequent epidermal exposure to the same Ag they are then released into the circulation and migrate to the site of Ag deposition. In fact, CH responses can be passively transferred to naive mice with immune lymph node cells (LNC). Recent studies of systemic passive transfer showed that the production of IL-4 by transferred LNC in the recipient mice is critical for systemic transfer (2). These results suggested the essential role of type 2 cytokines at the effector phase of CH and challenged the traditional paradigm that CH is relegated only to type 1 CD4+ T cells. Indeed, on the basis of adoptive transfer experiments with hapten-sensitized LNC, two groups of investigators (2, 3) have advocated that γδ+ T cells are required to assist these Ag-specific αβ+ CH effector T cells; in contrast, other groups of investigators (4, 5) suggested that CD8+ T cells mediate CH and that the magnitude of the response is inhibited by CD4+ T cells. However, interpretation of these studies depends heavily on inductive logic; that is, investigators have to assume that Ag-primed T cells in draining LN behave like those of the same phenotype in inflammatory skin sites, although immune responses in the LN may not accurately reflect the pattern of those present in the skin site. The choice of the regional draining LN as a source of these Ag-specific effector T cells would be based largely on the practical consideration of being able to easily recover, identify, and separate responding cells at various time points after antigenic challenge. Thus, there is little information available on the differences between LN T cells and skin-infiltrating T cells. Moreover, although CH is a dynamic process, and the balance among various cell types probably changes with time, much of previous efforts in studies on CH have focused largely on immune responses that occur at 24–48 h after challenge in the acute phase. In this regard, previous work from this laboratory has established that repeated application to the same skin site of hapten results in a shift in the time course of Ag-specific CH responses from a typical delayed-type hypersensitivity (DTH) response to an early-type response in a site-restricted fashion (6), and that a shift in cutaneous cytokine expression from a type 1 to a type 2 profile...
is responsible for the development of an early-type response at the sites of repeated hapten application (7). Our experimental design was chosen so as to mimic the in vivo situation more clinically relevant, because patients with chronic allergic contact dermatitis and atopic dermatitis are likely to be exposed repeatedly to the sensitizing Ags.

In the present study we have asked whether repeated elicitation of CH could skew T cell development to the type 2 phenotype in the regional draining LN as previously demonstrated in the skin sites. Our results have shown some interesting differences in the kinetics and profile of cytokine expression between the skin sites and the draining LN samples: while in the skin sites repeated elicitation of CH induced the development of highly polarized type 2 responses, in the corresponding draining LN the shift to type 2 cytokine production was also observed, but more mixed patterns of responses were induced. In vitro stimulation of the LNC from mice repeatedly exposed to the hapten produced markedly lower levels of type 1 cytokines and higher levels of type 2 cytokines compared with those from mice in the acute phase. However, despite their distinct cytokine profiles in vivo and in vitro, there was no difference in the ability of such polarized and functionally opposing populations of LNC obtained from mice in different phases to mediate systemic passive transfer of DTH responses in naive recipients. A type 2-rich cutaneous milieu established by repeated elicitation of hapten was required for successful passive transfer of an early-type response by the LNC. These results indicate that the local microenvironment in chronic inflammation represents a complex milieu, in which both T cells recruited from the draining LN and those strategically taking up residence in the lesional skin are involved. This complex milieu will not be recognized when only pure LNC populations are studied.

Materials and Methods

Mice

Male BALB/c mice were obtained from Charles River Japan (Kanagawa, Japan) and were used at 6–8 wk of age.

Contact-sensitizing (CS) agents

2,4,6-Trinitro-1-chlorobenzene (TNCB) was obtained from Tokyo Kasei (Tokyo, Japan); 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OX) was obtained from Sigma (St. Louis, MO). Each CS agent was dissolved in acetone to 1% solutions and used for sensitization and elicitation.

Sensitization and elicitation procedure

BALB/c mice were sensitized by an epicutaneous application of 20 μl of 1% TNCB or OX solution to the right ear, as previously described (6, 7). In the experiment of the acute phase of CH, 7 days later (day 0), sensitization to TNCB or OX was elicited with 20 μl 1% TNCB or OX solution to the right ear, as previously described (6, 7).

Ear thicknesses were measured with a dial thickness gauge (Ozaki Seisakusho, Tokyo, Japan) under light ether anesthesia. The ear swelling responses elicited by epicutaneous application of the CS agent on days 0–24 or 0–40 were identical with those reported by Murphy (9). Cycle conditions were 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. This process was repeated for 18 cycles with G3PDH primers; for 22 cycles with TGF-β1 primers; for 26 cycles with IL-1β, IL-1α, IL-4, IL-6, IL-10, IFN-γ, TNF-α, TGF-β1, GM-CSF, and G3PDH were purchased from Clontech (Palo Alto, CA). PCR primer sequences for IL-12 p35 and IL-12 p40 were identical with those reported by Murphy (9).

Reverse transcription

PCR was performed using a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). cDNA (2 μl) was amplified in a 20-μl reaction volume containing 10 nmol Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 μM of each dNTP, 0.5 μM primers, and 1 U Taq polymerase (Takara Shuzo) overlaid with mineral oil. PCR primers for IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IFN-γ, TNF-α, TGF-β1, GM-CSF, and G3PDH were purchased from Clontech (Palo Alto, CA). PCR primer sequences for IL-12 p35 and IL-12 p40 were identical with those reported by Murphy (9). Cycle conditions were 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. This process was repeated for 18 cycles with G3PDH primers; for 22 cycles with TGF-β1 primers; for 26 cycles with IL-1β, IL-1α, IL-4, and IL-10 primers; for 28 cycles with IL-12 p35 and IL-12 p40 primers; for 30 cycles with IL-2, IL-3, IL-10, IFN-γ, and TNF-α primers; and for 32 cycles with IL-6 and GM-CSF primers. After the final cycle, the temperature was maintained at 72°C for 7 min. PCR products were electrophoresed through 1.5% agarose gels in TAE buffer containing ethidium bromide, and the gels were photographed under UV light. Both positive and negative controls were included in each assay to confirm that only cDNA PCR products were detected and that none of the reagents was contaminated.

Semiquantitative PCR

The PCR products were electrophoresed through 2.5% agarose gels in TAE buffer. The gels were dried, and the radioactive signal–specific bands were quantified by a Bioimaging analyzer (BAB 2000, Fuji Photo Film, Tokyo, Japan). In semiquantitative experiments, PCR was set up as described above, except that the reaction mixture contained 20 μCi/ml of [32P]dCTP as tracer, and amplifications were run for 18 cycles with G3PDH primers, for 24 cycles with IL-4 and IL-10 primers, and for 26 cycles with IFN-γ and IL-12 p35 primers. In initial experiments we established standard curves for IL-1, GM-CSF, IL-12 p35, and IL-12 p40 sequences for IL-12 p35 and IL-12 p40 were identical with those reported by Murphy (9). Cycle conditions were 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. This process was repeated for 18 cycles with G3PDH primers; for 22 cycles with TGF-β1 primers; for 26 cycles with IL-1β, IL-1α, IL-4, and IL-10 primers; for 28 cycles with IL-12 p35 and IL-12 p40 primers; for 30 cycles with IL-2, IL-3, IL-10, IFN-γ, and TNF-α primers; and for 32 cycles with IL-6 and GM-CSF primers. After the final cycle, the temperature was maintained at 72°C for 7 min. PCR products were electrophoresed through 1.5% agarose gels in TAE buffer containing ethidium bromide, and the gels were photographed under UV light. Both positive and negative controls were included in each assay to confirm that only cDNA PCR products were detected and that none of the reagents was contaminated.

Measurement of in vitro cytokine production by LNC

Single-cell suspensions were prepared by gentle teasing from the draining LN collected from three to five mice per experiment in either the acute (day 0) or chronic (days 24–30) phase of CH and used for cytokine production assays. The LNC suspensions were cultivated at a concentration of 2–50×10^5 cells/well in RPMI 1640 tissue culture medium supplemented with 10% endotoxin-free, heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5 mmol/L 2-ME in the presence or the absence of either Con A (2 μg/ml) or 2,4,6-trinitrophenyl (TNP)-coupled spleen cells. TNP-coupled spleen cells (TNP-SC) were prepared by coupling 2,4,6-trinitrobenzene sulfonic acid (Tokyo Kasei) to syngeneic spleen cells as described (10). Briefly, 1×10^7 spleen cells (1×10^7/ml) were incubated with 3 μg/ml of 2,4,6-trinitrobenzene sulfonic acid at 25°C for 15 min, washed three times with medium, pelleted by centrifugation, and treated with mitomycin C. For cytokine production analysis, supernatants from replicate
cultures, usually three to five wells, were collected after 24–72 h and pooled. Because our preliminary experiments showed that the most optimal cytokine production by LNC was observed in the culture supernatants harvested at 48 h, supernatants for cytokine determination were harvested after 48 h of culture. For blocking experiments with mAbs against IL-4 and IL-10, anti-IL-4 mAb (clone 11B11, PharMingen, San Diego, CA), anti-IL-10 mAb (clone JES5-2A5, PharMingen), or a combination of both were added at the beginning of the culture at a concentration of 10 μg/ml. IL-2, IL-4, IL-10, and IFN-γ concentrations in the supernatants were quantified by ELISA kits (PerSeptive Diagnostics, Cambridge, MA) according to the manufacturer’s instructions.

Flow cytometry

Single-cell suspensions were prepared from draining auricular LN collected from three to four mice per experiment in either the acute (day 0) or chronic (days 24–30) phase of CH or from normal mice. The following procedures were conducted for two-color flow cytometric analysis as described previously (11). After two washes with PBS containing 1% heat-inactivated FCS and 0.1% sodium azide, the suspensions were incubated with appropriately diluted FITC-labeled mAb on ice for 30 min, followed by two washes. Appropriately diluted PE-conjugated mAb and propidium iodine (2 μg/ml) were added for an additional 30 min, followed by two washes. Dead cells were excluded by the propidium iodide staining. Viable cells were analyzed by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). The following antibodies were used: FITC- or PE-conjugated anti-CD3ε (clone 145-2C11, hamster IgG, PharMingen), FITC- or PE-conjugated anti-TCRβ (clone H57-697, hamster IgG, PharMingen), FITC- or PE-conjugated anti-TCRγδ (clone GL3, hamster IgG, PharMingen), FITC- or PE-conjugated anti-CD4 (clone RM4-5, rat IgG2a, PharMingen), FITC- or PE-conjugated anti-CD8 (clone 53-6.7, hamster IgG, PharMingen), FITC-labeled anti-B220 (clone RA3-6B2, PharMingen), and FITC-labeled anti-NK1.1 (clone PK136, PharMingen).

Adoptive cell transfer of CH

Sensitized mice were killed by cervical dislocation on day 3 (4 days after contact sensitization with either TNCB or OX; acute LNC) or day 24 (2 days after the final elicitation with either TNCB or OX; chronic LNC). The draining LN were removed, and single-cell suspensions were prepared by passing through nylon mesh and then were washed twice with PBS. The cells resuspended in PBS were injected i.v. at 3 × 10^7 cells/recipient into either naive syngeneic recipients or sensitized recipients repeatedly elicited with the CS agent. The recipient mice were elicited with the CS agent 24 h after adoptive cell transfer.

Results

Kinetics of cytokine mRNA expression in the draining LN during distinct phases of CH

We previously demonstrated that the pattern of cytokine mRNA expression in tissue samples at various time points after elicitation with TNCB varied depending on the phases of CH (7). In acute skin lesions taken from mice that were sensitized with TNCB and elicited 7 days later on the originally sensitized right ear, type 1 responses predominated, whereas the chronic skin lesions of mice similarly sensitized and repeatedly elicited with TNCB for 24 days expressed predominantly type 2 cytokines and a loss of type 1 cytokine production. To investigate whether similar type 1/type 2 polarization could be seen in the draining LN in the acute and chronic phases of CH, the draining LNs were collected at various time points after each elicitation with TNCB in the two distinct phases of CH for the quantification of mRNA for 13 cytokines. As described previously in skin samples (7), a specific product of the predicted size was detected in all samples for each of the cytokines tested. Different kinetic profiles of cytokine mRNA expression were observed after elicitation with TNCB in the draining LN in the chronic phase compared with the acute phase (data not shown). A dominance of type 1 cytokines in the acute lesions and that of Th2 cytokines in the chronic lesions was clearly observed in these LN samples as previously described with skin samples (7).

Of the 13 cytokines tested, mRNA expression for IL-2 and IFN-γ that typify type 1 cytokines and for two typical type 2 cytokines, IL-4 and IL-10, was subsequently analyzed by semiquantitative PCR method as described previously (7). The relative amounts of cytokine mRNAs present in the draining LN taken from the acute and chronic lesions were expressed as the value relative to amounts of G3PDH mRNA. This approach allowed comparison of the expression levels between the LN populations obtained in the two phases regardless of the absolute number of cells used for RNA isolation. Fig. 1 shows representative autoradiograms highlighting mRNA expression for these cytokines in the draining LN and the corresponding skin samples. In acute LN (Fig. 1), significant elevations (compared with levels before elicitation) in IFN-γ, IL-2, and IL-4 were found at 3 h. IFN-γ expression peaked at 6 h, remained elevated at 9–12 h, and later with dropped, while IL-2 expression started to decrease at 6 h, but did not return to levels before elicitation by 24 h. Of note, the expression of IL-4 was synchronous with that of type 1 cytokines at early times, a finding not observed in the corresponding skin samples. IL-4 expression showed the same pattern as that of IL-2, with peak expression at 3–6 h. However, unlike IL-2 expression, at 12 h IL-4 levels rapidly decreased to levels below those observed before elicitation, although a transient re-increase in the expression was observed at 24 h. In contrast, IL-10, another type 2 cytokine, was either undetectable or very low throughout the observation period. Although there was a similar tendency for the predominance of type 1 cytokine in the acute LN sample, the pattern as well as the levels of mRNA expression observed in the draining LN were different from those found in the corresponding skin samples. In the skin samples, kinetic profiles of IL-4 expression were synchronous with those of IL-10 expression, and mRNA levels for type 2 cytokines were up-regulated at later times (12–24 h); in contrast, in the acute LN, IL-4 expression increased sharply at 3 h, and the levels of IL-10 were almost undetectable.

In chronic LN (Fig. 1), minimal IFN-γ mRNA expression was found, and a slight up-regulation of IL-2 expression was detected at 1–6 h. Before elicitation with TNCB, IL-4 mRNA was abundantly present, as observed in the corresponding skin samples, and was minimally up-regulated at 1h. After 1 h of elicitation, IL-10 levels were rapidly increased, remained elevated at 3 h, and decreased to undetectable levels by 24–48 h. The overall kinetic profiles of cytokine mRNA expression detected in the LN samples of the chronic phase were similar to those detected in the corresponding skin samples, except for the absence of rapid up-regulation in IFN-γ expression detected in the skin at 1–3 h. To confirm Ag specificity in cytokine mRNA expression, groups of TNCB-sensitized mice were subsequently elicited with TNCB for 24 days, and the TNCB-treated ears were treated with a primary application of OX, an antigenically distinct CS agent (designated chronic OX). These experimental mice were compared with two groups of control mice: control unprimed mice were treated with a primary application of TNCB (designated normal), and mice sensitized with TNCB were elicited with TNCB 7 days later in the same way as acute, but to the nontreated left ear (designated primed). In these experiments, the draining LN and skin samples were removed from the same mice at various time points after TNCB or OX application and were subjected to semiquantitative PCR analysis of cytokine mRNA expression. As shown in Figs. 1 and 2, in the draining LN from normal mice peak levels of IFN-γ and IL-2 were seen at later times (24–48 h), which plausibly represents the priming response in vivo. This priming response was never observed in the corresponding skin samples. Of interest, however, primed lesional skin showed a similar pattern of up-regulation for IFN-γ and IL-2 that peaked at 12 h; these kinetics were comparable to those observed in the corresponding LN samples. These results are consistent with a scenario that primary sensitization of naive T cells...
after TNCB application occurs initially within the draining LN, but not in the skin, and that after a second application of TNCB to the nontreated left ear, part of these sensitized T cells with a type 1 phenotype are rapidly recruited to the skin site, while part of those remain in the draining LN. This priming response was also marginally observed in the draining LN taken from mice repeatedly treated with TNCB after OX application (chronic OX), although it was very low compared with that observed in the normal mice treated with TNCB application. Significant up-regulation of IL-10 expression was not observed in the draining LN taken from those mice after a primary application of OX (chronic OX), indicating that up-regulation of IL-10, and possibly IL-4, observed in the draining LN of the chronic phase (chronic) is an Ag-specific immunologic phenomenon.

Taken together, these data may serve to indicate that during the induction and evolution of CH the profile of cytokine gene expression in the draining LN proceeds from a typical type 1 phenotype to a type 2-dominated phenotype in a less stringent manner than that in the corresponding skin samples. The profile of cytokine gene expression appears to proceed in the following order: first, the most polarized type 1 responses (abundant IFN-γ and IL-2 at later time points, and no IL-4 and little IL-10) in the normal LN; second, polarized type 1 responses (abundant IFN-γ and IL-2 at later time points and little IL-4 and IL-10) in the primed LN; third, type 1-dominated, but more mixed, responses (abundant IFN-γ, IL-2, and IL-4, and little IL-10) in the acute LN; and finally, type 2-dominated responses (little IFN-γ and IL-2, and abundant IL-4 and IL-10) in the chronic LN. Interestingly, this pattern of cytokine expression was noted in each sample obtained before elicitation (note the background cytokine expression at 0 h).

**FIGURE 1.** Autoradiograms of the PCR products for each cytokine obtained by semiquantitative PCR in the draining LN and the corresponding skin samples taken from the same mice at various time points after TNCB (unless otherwise indicated) or OX (indicated) application in normal, primed, acute, and chronic (OX) mice. Normal mice represent those unprimed, and primed mice represent those sensitized with TNCB 7 days before. At the point indicated with 0, the four groups of mice (normal, primed, acute, and chronic) were elicited with TNCB application to either the right ear (normal, acute, and chronic) or the nontreated left ear (primed), and chronic (OX) mice were treated with a primary application of OX to the TNCB-treated right ear. In these experiments, LN and skin samples for cytokine determinations were obtained from the LN draining the site and from the site, respectively, which were treated with either TNCB (normal, primed, acute, and chronic) or OX (chronic [OX]) at 0 h. Samples at 0 h represent those taken immediately before final elicitation. For the sake of the point-by-point comparison of these cytokine data in the draining LN and the corresponding skin samples, part of the data in the skin sample is reproduced from our previous paper (7). The data shown are representative of three independent experiments.

**FIGURE 2.** A graphic representation of relative levels of cytokine mRNA expression at various time points after TNCB application in normal (■), acute (○), and chronic (●) LNs. The levels of mRNA expression for each cytokine are expressed as the value relative to amounts of mRNA for G3PDH, as described in Materials and Methods. Each time point represents the mean ± SD of three independent experiments.

**In vitro cytokine production by the LNC**

To determine whether the pattern of in vivo cytokine mRNA expression by these LN observed after TNCB application could be also observed during in vitro culture with mitogen or Ag, cells
isolated from the LN draining the sites of TNCB application were stimulated with mitogen or specific Ag and were used for in vitro cytokine production assays. We initially reasoned that compared with polyclonal activators, specific Ag-stimulation probably reflects cytokine patterns that are more representative of in vivo cytokine production in response to hapten application. Therefore, in most experiments in vitro cytokine production by LNC was determined on supernatants generated by those stimulated with either mitogen or specific Ag, TNP-SC. Our experiments indicated that mitogen stimulation yielded a response pattern similar, but not identical, to that seen after stimulation with specific Ag. Compared with stimulation of LNC with TNP-SC, however, not only did mitogen stimulation reproducibly generate 2- to 3-fold greater levels of IFN-γ, IL-4, and IL-10, but the pattern of cytokine production more accurately mimicked the in vivo cytokine mRNA expression in the draining LN after TNCB application. It is well recognized that the frequency of Ag-specific T cells in lesional skin and draining LN is extremely low even at the peak of the CH response, and bystander activation of non-Ag-specific T cells is a substantial component of the T cell response in vivo. Thus, the local environment after TNCB application would represent a complex milieu of polyclonal responses of Ag-specific T cells and bystander activation of non-Ag-specific T cells. We therefore reasoned that in vitro cytokine production by LNC stimulated with mitogen would more closely mimic events occurring in the LN in vivo, rather than by LNC stimulated with TNP-SC. Furthermore, cytokine levels in culture supernatants of Ag-stimulated LNC were too low to determine accurately the effect of anti-IL-4 mAb and/or anti-IL-10 mAb, which was important for the next series of experiments. Thus, the primary reason for studying in vitro cytokine production by mitogen-stimulated LNC was that it was thought to reflect more closely what happens in the draining LN when mice sensitized with TNCB are exposed to TNCB.

Consistent with in vivo results, a very high production of IL-2 and IFN-γ was found in culture supernatants of mitogen-stimulated LNC in the acute phase, much higher than that induced in those of LNC in the chronic phase. The quantitative difference in IFN-γ production by LNC between the acute and chronic phases was even more striking than that in the IL-2 production (Figs. 3 and 4). In contrast, type 2 cytokine responses by the LNC were much less divergent: nevertheless, the chronic LNC made significantly more IL-4 and IL-10 than the acute LNC, although the difference was less evident than those observed from our in vivo data (Figs. 1 and 2). When graded numbers of the LNC were stimulated with mitogen, more dramatic differences in cytokine production were noted, as shown in Fig. 3. These results confirmed our conclusion that the acute LNC are skewed toward production of type 1 cytokines, whereas the chronic LNC are skewed toward type 2 responses.

However, it remained unknown whether this reduced type 1 cytokine production of chronic LNC is due to lower frequencies of type 1 cells or an inhibitory effect on type 1 cytokine production by IL-4 and/or IL-10 endogenously produced during in vitro stimulation. It was important, therefore, to determine whether addition of anti-IL-4 mAb and/or anti-IL-10 mAb would restore type 1 cytokine production by chronic LNC. To this end, we examined whether neutralization of endogenous IL-4 and/or IL-10 by these mAbs during in vitro culture could alter the pattern of in vitro cytokine production by these LNC. As shown in Fig. 4, anti-IL-4 mAb treatment increased increases in IL-2, but not IFN-γ, production by chronic LNC, whereas anti-IL-10 mAb had the opposite effect, augmenting IFN-γ production by 2-fold, but had no effect on IL-2 production. Unlike our expectation, however, the capacity of the chronic LNC to produce type 1 cytokines was restored to some extent, but did not reach levels comparable to those in acute LNC even in the presence of both anti-IL-4 and anti-IL-10. This observation indicates a lower frequency of type 1 cells in the chronic LNC population. In contrast, anti-IL-4 and anti-IL-10 had only marginal, if any, enhancing effects on IL-2 and IFN-γ production by acute LNC. Their enhancing effect on type 1 cytokine production by acute LNC was only apparent when both mAbs were used in a combination. These findings are consistent with in vitro and in vivo cytokine data and lend further support to the idea that patterns of cytokine production are profoundly different in the LNC between the acute and chronic phases of CH.
Phenotypic difference in acute vs chronic LNC

To examine whether the differences in the profile of cytokine production between the acute and chronic LNC could reflect the phenotypic differences, cell suspensions of the draining LNC were analyzed by flow cytometry after staining with various mAbs. A summary of the staining data from multiple experiments is presented in Table I. The phenotypic analyses were also performed on the LNC from normal mice as a control. The majority of the LNC in the three different populations expressed CD3 and TCR-\(\alpha\beta\): the percentages of CD3\(^+\), TCR-\(\alpha\beta\)\(^+\), CD4\(^+\), and CD8\(^+\) cells in the total numbers of draining LNC were basically similar between the acute and chronic LNC, although the absolute numbers of the yield per mouse of lymphocytes recovered from the draining LN in the chronic phase were 5- to 6-fold greater than those in the acute phase. The percentages of Thy 1\(^+\), CD3\(^+\), CD4\(^+\), CD8\(^+\), and \(\alpha\beta\)\(^+\) cells in normal LNC were greater than those in the acute and chronic LNC, whereas the normal LNC contained smaller percentages of \(\delta\)^\(\delta\), NK1.1\(^+\), and Ia\(^+\) cells. Collectively, these data indicate that the distinct differences in the profile of cytokine production between the acute and chronic LNC cannot be simply explained by the phenotypic differences. Nevertheless, one may argue that the differences in the cytokine profile observed among these LN preparations are due in part to alterations in other cell populations, such as eosinophils, neutrophils, and dendritic cells, which were unable to be examined by flow cytometry, because all these populations secrete cytokines that could profoundly affect the pattern and kinetics of cytokine production by T cells. However, exhaustive histologic examinations of the draining LN in the acute and chronic phases of CH provided little or no evidence of recruitment of eosinophils, neutrophils, and mast cells to the draining LN (data not shown). It is, therefore, possible that dendritic cells are candidates for directing T cells toward a defined phenotype by providing not only the ligands for the TCR and costimulatory molecules but also the necessary cytokines.

Adoptive transfer of type 1/type 2 polarized LNC

In the previous study using passive local transfer of polyclonal murine type 1 and type 2 cells obtained after short or long term culture in vitro, Müller et al. (12) demonstrated that type 1 cells induced a typical DTH response, peaking at 24–48 h, type 2 cells

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**Table I. Phenotypic analysis of acute and chronic LNC**

<table>
<thead>
<tr>
<th></th>
<th>% of Total LNC (^a)</th>
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<tbody>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Thy 1(^+)</td>
<td>65.7 ± 5.2</td>
</tr>
<tr>
<td>CD3</td>
<td>66.3 ± 5.6</td>
</tr>
<tr>
<td>CD4</td>
<td>49.9 ± 2.7</td>
</tr>
<tr>
<td>CD8</td>
<td>15.8 ± 1.6</td>
</tr>
<tr>
<td>TCR-(\alpha\beta)</td>
<td>67.6 ± 3.2</td>
</tr>
<tr>
<td>TCR-(\gamma\delta)</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>I-A(^d)</td>
<td>32.2 ± 6.0</td>
</tr>
<tr>
<td>NK1.1</td>
<td>1.3 ± 0.6</td>
</tr>
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</table>

| Acute\(^c\) |                        |
| Thy 1\(^+\) | 49.5 ± 4.2             |
| CD3       | 51.6 ± 5.6             |
| CD4       | 33.4 ± 4.1             |
| CD8       | 12.4 ± 0.6             |
| TCR-\(\alpha\beta\) | 50.3 ± 4.9             |
| TCR-\(\gamma\delta\) | 4.9 ± 5.2             |
| I-A\(^d\)  | 47.1 ± 5.7             |
| NK1.1     | 3.2 ± 0.8              |

| Chronic\(^c\) |                        |
| Thy 1\(^+\) | 46.4 ± 3.6             |
| CD3       | 55.8 ± 5.9             |
| CD4       | 31.8 ± 2.9             |
| CD8       | 11.6 ± 1.9             |
| TCR-\(\alpha\beta\) | 50.4 ± 6.1             |
| TCR-\(\gamma\delta\) | 2.9 ± 0.4             |
| I-A\(^d\)  | 42.4 ± 6.7             |
| NK1.1     | 2.2 ± 0.1              |

\(^a\) LNC suspensions were prepared from the draining LN collected from at least three mice/group.

\(^b\) Data are expressed as the mean percentages of cells expressing each Ag ± SD of three independent experiments.

\(^c\) Acute LNC were obtained 7 days after sensitization with TNCB (day 0), and chronic LNC were obtained 2 days after completion of the 30-day repeated application protocol (day 30).

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**FIGURE 4.** Effect of anti-IL-4 and anti-IL-10 mAb on cytokine production by acute and chronic LNC stimulated with mitogen. Data shown are the mean of triplicate wells ± SD and are representative of three separate experiments. Our preliminary experiments demonstrated that concentrations of anti-IL-4 and anti-IL-10 used were sufficient to completely neutralize endogenous IL-4 and IL-10, respectively. Thus, complete inhibition of IL-4 and IL-10 production by anti-IL-4 and anti-IL-10, respectively, was not examined in additional experiments. N.D., not determined.
induced an early-type response that was maximal at 6 h and there-
after fell toward baseline at 48 h, and a mixture of type 1 and type
2 cells induced a swelling with features of both type 1- and type
2-mediated responses. We, therefore, analyzed the ability of acute
and chronic LNC to transfer a DTH response or an early-type
response by injecting the cells i.v. into nonimmune syngeneic mice
never exposed to TNCB or OX; those recipients were subsequently
challenged with hapten application. For this purpose, TNCB- or
OX-sensitized, acute LNC were taken 4 days after sensitization
with TNCB or OX, respectively, and chronic LNC were prepared
from mice repeatedly treated with TNCB or OX 2 days after com-
pletion of the 30-day repeated application protocol. The results of
flow cytometry of the transferred cell types were consistent with
those described above in the phenotypic analysis of the draining
LNC. As controls, PBS, instead of normal LNC, was infused into
nonimmune syngeneic mice, because the draining LN of nonsens-
sitized mice were too small to yield numbers of lymphocytes suf-
ficient for transfer experiments. As shown in Fig. 5A, passive
transfer of TNCB-sensitized, chronic LNC into nonimmune syn-
genetic recipients resulted in transfer of Ag-specific DTH respon-
siveness that was comparable in kinetics and in magnitude to that
seen with transfer of TNCB-sensitized acute LNC when assayed 1
day after transfer of cells. The kinetics and magnitude of the ob-
served ear swelling response were similar to those observed in
TNCB-sensitized mice elicited on the nontreated ear with a sec-
ondary application of TNCB under conditions where about 3 × 10^7
LNC were infused. Similar results were obtained by injecting
OX-sensitized, acute or chronic LNC i.v. into nonimmune syngen-
neic mice followed by epicutaneous challenge with OX, as shown
in Fig. 5B. The transfer was Ag specific, because passive transfer
of the draining LNC from TNCB-sensitized mice led to no signif-
icant swelling when challenged with OX and vice versa. These
results suggest that chronic LNC may contain sufficient numbers of
type 1 cells for systemic transfer of DTH despite the overall po-
larizaton to type 2 as evidenced by in vivo and in vitro cytokine
production. Thus, the development of an early-type response in the
ears repeatedly treated with hapten could not be explained by the
ultimate dominance of type 2 cells in the draining LNs, because
acute and chronic LNC had comparable abilities to transfer DTH,
which is thought to be the sole property of type 1 cells. We, there-
fore, reasoned that the cutaneous cytokine milieu, rather than the
type 1/type 2 polarization in the draining LNC, would determine
whether a DTH response or an early-type response would develop
at the sites.

To test this possibility, we investigated whether adoptive trans-
fer of OX-sensitized, acute LNC into mice repeatedly elicited with
TNCB could elicit an early-type reaction peaking at 6 h in a site-
restricted manner. As shown in Fig. 6B, OX-sensitized, acute LNC
taken 4 days after sensitization with OX were able to transfer DTH
responses that peaked at 24 h, when recipient mice were elicited
with OX on the nontreated left ear. In contrast, these OX-sensi-
tized, acute LNC induced a significant early-type response that was
maximal at 6 h and waned to near baseline levels by 24 h when the
repeatedly TNCB-treated right ears of recipient mice were elicited
with OX (Fig. 6A). Similar results were obtained with OX-sensi-
tized, chronic LNC (data not shown). Taken together, these results
indicate that the cutaneous cytokine milieu established by repeated
application of hapten would favor the development of an early-
type response by triggering type 2-dominated responses and by
inhibiting potentially detrimental type 1 responses.

**Discussion**

In this study we have demonstrated that the kinetics and pattern
of cytokine production in draining LNs do not necessarily correspond
to those found in skin samples regardless of the phase of CH. Of
interest was the marked differences in the expression levels of
IFN-γ between the skin sample and the draining LN. Transcrip-
tional activation of IFN-γ genes in the draining LN occurred at
lower levels than in the corresponding skin sites during the same
period regardless of the phase of CH. Because previous in vitro
studies demonstrated that IL-4 inhibits priming for IFN-γ produc-
tion by CD4^+ T cells (13), this difference may be explained by an
increase in IL-4 gene transcription at early time points demon-
strated in the draining LN; IFN-γ production by activated LNC
may have been suppressed by in vivo exposure to high levels of
IL-4 released early in the draining LN in response to TNCB
application.

The finding that cytokine mRNA expression in the draining LN
was rapidly up-regulated after TNCB application over a time
course indistinguishable from that in the skin samples was some-
what surprising, as we predicted that up-regulation of cytokine
mRNA expression in draining LN would follow that in the skin
site with delay. The mechanism of recruitment of Ag-specific T
cells present in draining LN into peripheral challenge sites upon
the transmission of Ag signals is not fully understood; it remains
largely unknown whether the Ag-specific T cells are initially ac-
ivated in local draining LN and then recruited into the skin site
where significant amounts of Ag persist, or whether those nonspe-
cifically recruited into the site are selectively activated and pref-
entially retained there. Given the rapid up-regulation of cytokine
mRNA following hapten application in both skin and LN samples, these T cells would be activated in skin sites and draining LN, respectively. The kinetics of cytokine mRNA expression in the draining LN in the acute phase, however, were quite different from those in the corresponding skin samples; particularly with respect to type 2 cytokines, the great disparity in cytokine mRNA expression was noted between skin sites and draining LN. This disparity may well reflect the differing capacities of type 1 and type 2 cells to migrate into the skin sites. In the acute phase Ag-specific T cells with a type 1 phenotype predominantly present in the draining LN may initially migrate into the sites, and those with a type 2 phenotype may be subsequently recruited into the sites to subserve an effector function that reduces the tissue-damaging effects of the type 1 responses as a self-protection mechanism. In this regard, recent studies have demonstrated that murine type 1 cells migrate into inflamed sites of a DTH reaction for the skin much better than type 2 cells do, and that P-selectin glycoprotein ligand-1, the major P-selectin ligand on type 1 cells, is relevant for the entry of these cells into inflamed skin (14, 15). Our finding, therefore, could be interpreted to indicate that efficient recruitment and preferential retention of type 2 cells with reduced ability to migrate into the skin may require additional factors that are probably delivered as a consequence of local activation of type 1 cells. Considering our finding that adoptively transferred acute LNC as well as the chronic LNC migrated into the skin sites established by repeated hapten application and induced an early-type response that is thought to be mediated by type 2 cells, these factors would be abundantly present in the chronic, but not the acute, lesions. This view is also supported by the observation that the migration of type 2 cells into inflamed sites was comparable to that of type 1 cells in a type 2-dominated allergic response established by repeated application of aerosolized Ag (14).

An unpredictable finding in this study was that the ability of TNCB-sensitized, chronic LNC to transfer Ag-specific DTH responses by injecting the cells systemically into naive recipients was comparable to that of TNCB-sensitized, acute LNC. In view of the much lesser ability of type 2 cells to enter the inflamed skin (14, 15), a reasonable explanation for this finding is that Ag-specific type 2 cells present in chronic LNC may not efficiently enter the skin sites where a cytokine milieu does not allow type 2 cells to extravasate from the bloodstream and enter the skin sites because of the defective ability of type 2 cells to bind P-selectin (14). This explanation is likely because the ability of type 2 cells to transfer an early-type response to recipient mice was only demonstrated by local injection of these cells previously activated in vitro (12). However, we could not directly prove this possibility because at this time it is technically not possible to determine whether type 2-polarized, chronic LNC could mediate a local passive transfer of an early-type response by injecting them locally into naive recipients; background swelling induced by local injection of LNC per se made it difficult to detect Ag-specific ear swelling responses, particularly at early time points after local injection. A second possible explanation for our failure to demonstrate systemic transfer of an early-type response by the chronic LNC is that type 2 cells present in the LNC can migrate into the site of Ag challenge, but the cutaneous cytokine milieu in naive recipients may not enable their subsequent reactivation upon antigenic encounter. As previously suggested (16–18), the abundance of type 2 cytokines (IL-4 and IL-10) in vivo, either before or during antigenic challenge, would be essential for subsequent activation of type 2 cells. Support for this view is found in our observation that the type 1-dominated, OX-sensitized, acute LNC as well as the type 2-dominated, chronic LNC can induce an early-type response when transferred into mice repeatedly elicited with TNCB and elicited with OX on TNCB-treated right ear; type 1 cells abundantly present in the acute LNC would be prevented from expressing their potential to mount DTH responses in the type 2 cytokine-rich milieu established by repeated hapten application. Indeed, recent studies have provided evidence to indicate that endogenously produced IL-4 and/or IL-10 may act to down-regulate CH responses (19–21). In view of the negative effects of IL-10 and IL-4 on the ability of APC to prime type 1 cells (18), a functional APC population residing in the chronic skin lesions and LN might have a defective ability to support the priming of type 1 cells by exposure of APC to type 2 cytokines in vivo.

A recent paper by Zhang et al. (22) provided another mechanism for the predominance of type 2 cells in tissue microenvironments where repeated exposure to Ag occurs; they clearly demonstrated that type 1, but not type 2, cells undergo a rapid Fas/Fas ligand-mediated activation-induced cell death upon reactivation. Thus, the relative persistence of IL-2-producing type 1 cells in the chronic LN compared with those in the corresponding skin sites might be explained by assuming that factors that not only prevent apoptosis but also enable rescued cells to be restimulated without inducing activation-induced cell death could be rich in the chronic LN, but not in the skin sites chronically elicited with hapten.

Our finding that the capacity of chronic LNC to produce IFN-γ was not restored by neutralization of IL-4 and IL-10 with mAbs toward levels of acute LNC deserves some comment. Because maximally neutralizing concentrations of anti-IL-4 and anti-IL-10 mAbs were added to LNC cultures in the presence of Con A, it is unlikely that incomplete restoration of the capacity of the chronic LNC to produce IFN-γ was due to our use of insufficient concentrations of the mAbs. These considerations raise the alternative possibility that the poor IFN-γ production in the chronic LNC may not result from inhibition of type 1 cells by type 2 cytokines but from the limited numbers of IFN-γ-producing T cells present in the chronic LNC. Consistent with this idea is the present finding that IFN-γ mRNA expression was only marginally detected in chronic LN even at early time points (Fig. 2C), while in chronic skin lesions an initial burst of IFN-γ mRNA comparable to that in acute skin lesions was observed at early time points (7). Thus, it remains inconclusive whether the decreased production of type 1 cytokines by chronic LN demonstrated both in vivo and in vitro could be attributed to inhibition of type 1 cells by type 2 cytokines endogenously produced or to lower frequencies of type 1 cytokine-producing cells in the population. To date, intracellular flow cytometric analysis of cytokine expression has been the most favorable approach to quantitate the number of cells in vivo primed to express type 1 cytokines. We are presently examining the pattern of cytokine production at the single-cell level in acute and chronic LNC using flow cytometric detection of intracellular cytokines.

In conclusion, the results of this study demonstrate that Ag-primed T cells in draining LN do not necessarily behave in the same way as those residing in the inflammatory skin sites. The cytokine microenvironment at the site of repeated antigenic challenge may have an important regulatory influence on efficient recruitment and preferential retention of Ag-primed T cells present in draining LN. Much remains to be answered, however, about a bidirectional communication between Ag-primed T cells present in inflammatory skin sites and those in the draining LN.

References


