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Intracellular expression of cytokines and granzyme B in auricular lymph nodes draining skin exposed to irritants and sensitizers

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ABSTRACT

The murine local lymph node assay (LLNA) has been extensively utilized to evaluate sensitizing chemicals. However, there have been some concerns that its use to discriminate between classes of chemicals is minimal. It is thus desirable to identify better or alternative immune endpoints with in LLNA itself. Here, we evaluated the protein and/or mRNA levels of cytokines and granzyme B (GzmB), a cytotoxic lymphocyte product, to discriminate between sensitizers and irritants and to characterize the chemical sensitizers when used as supplemental indicators in LLNA endpoints. For this, CBA/N mice were topically treated daily with a well-known chemical sensitizer such as a strong contact sensitizer 1-chloro-2,4-dinitrobenzene (DNCB), a skin contact sensitizer 2-phenyl-4-ethoxymethylene-5-oxazolone (OXA), and a skin or respiratory sensitizer toluene 2,4-diisocyanate (TDI), and the non-sensitizing irritants, croton oil (CRO) and nonanoic acid (NA), for 3 consecutive days. The protein and/or mRNA levels in auricular lymph nodes draining the ear skin were then analyzed by real-time RT-PCR and immunoassay.

The sensitizers, but not the irritants, evoked pronounced interleukin (IL)-2, IL-3 and IL-4 or interferon (IFN)-γ. Significantly, different sensitizers evoked different cytokine patterns of IL-4 and IFN-γ, as DNCB strongly up-regulated both IFN-γ and IL-4, OXA up-regulated IFN-γ strongly but IL-4 weakly, and TDI up-regulated IL-4 strongly but IFN-γ weakly. The sensitizers also strongly up-regulated GzmB mRNA, while the irritants had a much weaker effect. Thus, these cytokines and GzmB mRNA may be useful as additional endpoints for discriminating between irritants and sensitizers or contact and respiratory sensitizers in the LLNA.

1. Introduction

The murine local lymph node assay (LLNA) is a preferred method for assessing skin sensitization in chemical risk assessments (ICCVAM, 2001; OECD, 2002). In this test, the primary immune response to chemical is measured by determining the degree of ^{3}H-methyl thymidine incorporation into lymphocytes with supplemental indices of organ weight or cell counts in the local LNs draining the site of chemical application. Although LLNA has been validated as being suitable for hazard identification of skin-sensitizing chemicals (Dearman et al., 1999; Kimber et al., 2002), it is prone to give false positives for the chemicals that merely irritate the skin but not induce sensitizing effect. In addition, since different classes of chemical sensitizers induce divergent immune responses (Dearman et al., 1996a,b, 1999, 2003; Manetz and Meade 1999; Plitnick et al., 2002), a marker that can characterize chemical sensitizers according to the type of immune response they evoke would be also useful.

Many studies have tried to characterize chemical sensitizers according to the cytokine profiles they provoke (Dearman et al., 1996a,b, 1999, 2003; Hayashi et al., 2001; Manetz and Meade, 1999; Manetz et al., 2001; Plitnick et al., 2002; Vandebriel et al., 2000, 2003; Van Och et al., 2002). The development of immune responses is orchestrated by the activity of T helper 1 (Th1) and T helper 2 (Th2) cells and their cytokine products. Draining lymph nodes (LNs) have been shown to produce either a Th1-type cytokine pattern characterized by increased IFN-γ or a Th2-type cytokine pattern of high levels of IL-4 and IL-10 (Azam et al., 2005; Dearman et al., 2003; He et al., 2001; Kitagaki et al., 1999). There are contradictions in the availability of cytokine profiles produced by draining LN cells as an alternative LLNA readout. Early studies showed that IL-6 (Dearman et al., 1994), or IFN-γ and IL-12 (Dearman et al., 1999) are

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Table 1
Primer sequences used in real-time RT-PCR analysis

Gene symbol	Gene description		Primer sequence
Actb	Actin, beta, cytoplasmic	Sense	ATCTACGAGGGCTATGCTCTCC
		Antisense	CTGATCCACATCTGCTGGAAGG
IL-1α[(1)]	Interleukin 1 alpha	Antisense	CCTTCTATGATGCAAGCTATGG
		Sense	TAAACAGCTCTGGATAAGCAGC
IL-1β	Interleukin 1 beta	Antisense	GACGGACCCCAAAAGATGAAGG
		Sense	GAGGTGCTGATGTACCAGTTGG
IL-2	Interleukin 2	Antisense	GCCCAAGCAGGCCACAGAAT
		Sense	GGGCTTGTTGAGATGATGCTTTGA
IL-3[(2)]	Interleukin 3	Antisense	TTCTTGCCAGCTCTACCACC
		Sense	GGTTAGCACTGTCTCCAGATCG
IL-4[(3)]	Interleukin 4	Antisense	CTTCTTTCTCGAATGTACCAGG
		Sense	TTGCATGATGCTCTTTAGGC
IL-6	Interleukin 6	Antisense	ACTGATGCTGGTGACAACCACG
		Sense	TGGATGGTCTTGGTCCTTAGCC
IL-10[(2)]	Interleukin 10	Sense	TTACTGACTGGCATGAGGATCA
		Antisense	AAGTCTTCACCTGGCTGAAG
IL-12a[(2)]	Interleukin 12a	Sense	TGTCTTAGCCAGTCCCGAAACC
		Antisense	AGCTCAGATAGCCCATCACC
IL-12b	Interleukin 12b	Sense	GACATGGAGTCATAGGCTCTGG
		Antisense	TACGAGGAACGCACCTTTCTGG
IL-17[(2)]	Interleukin 17	Sense	CCAGGGAGAGCTTCATCTGT
		Antisense	AGGAAGTCCTTGGCCTCAGT
GM-CSF[(1)]	Colony stimulating factor 2 (granulocyte-macrophage)	Sense	GCAGAATTTACTTTTCCTGGGC
		Antisense	GTAACTTGTGTTTCACAGTCCG
G-CSF[(2)]	Colony stimulating factor 3 (granulocyte)	Sense	AGGCTCTATCGGGTATTTCCCC
		Antisense	TGGAAGGCAGAAGTGAAGGC
IFN-γ	Interferon gamma	Sense	GGCTGTTTCTGGCTGTTACTGC
		Antisense	ACTCCTTTTCCGCTTCCTGAGG
TNF-α[(2)]	Tumor necrosis factor	Sense	ATGAGCACAGAAAGCATGATCC
		Antisense	ACAAGCAGGAATGAGAAGAGG
GzmB[(4)]	Granzyme B	Sense	GGGGGCCCACAACATCAAAGAA
		Antisense	ACAAGCGGGCCTCCAGAATCC

The samples were denatured for 2.5 min at 95 °C and then subjected to 40 cycles of amplification and quantification (0.5 min at 57 °C, 1 min at 72 °C, and 1 min at 95 °C). This was followed by a melting point program (80 cycles of 10 s each, from 55 to 95 °C in 0.5 °C increments). The PCR products were annealed at: [(1)]55 °C, [(2)]56 °C, [(3)]53 °C or [(4)]59 °C.

unreliable and not sufficiently sensitive to serve as LLNA endpoints. However, more recently, when Azam et al. (2005) investigated whether IL-2 production could serve as a useful LLNA endpoint, IL-2 was elucidated quantitatively and qualitatively as efficient as the standard LLNA protocol. In addition to cytokine profiles, cytotoxic lymphocyte products such as granzyme B (GzmB) also arouse attention as supplemental indicators in the LLNA. GzmB has been used as a prognostic marker in early rheumatoid arthritis (Goldbach-Mansky et al., 2005) and is known to elicit cutaneous inflammation in atopic dermatitis (Tsuzaka et al., 2006). In recent studies, it was reported to be released by asthmatic patients after allergen challenge (Tschopp et al., 2006) and up-regulated in the LNs of 2,4-dinitrofluorobenzene-challenged rats (Hartmann et al., 2006). In our previous microarray analyses, GmzB expression was significantly up-regulated in the auricular LNs of sensitizer-treated mice (unpublished data). The present study thus approached to GzmB with expectation that it may be associated with chemical-induced contact hypersensitivity.

In the present study, to refine the LLNA for the differentiation and characterization of chemical-induced allergic responses, we investigated the protein and/or mRNA levels of various cytokines and GzmB in ear skin-draining auricular LNs of mice exposed to known sensitizers and irritants and examined their availability as supportive indicators for LLNA.

2. Materials and methods

2.1. Animals

SPF-maintained 7-week-old female CBA/N mice (SLC, Hamamatsu, Japan) were acclimatized for a week before the experiments. Mice were housed three to four per cage in polycarbonate cages with sterilized softwood bedding and fed commercial pellets (Purina, Dageon, Korea) and water *ad libitum*. The environment was maintained at 23 ± 2 °C with a relative humidity of 55 ± 10% and a 12 h-light/dark cycle.

2.2. Chemicals

All chemicals tested were of reagent grade or higher and were purchased from Sigma–Aldrich Chemicals (St. Louis, MO). The test chemicals included three kinds of sensitizers 1-chloro-2,4-dinitrobenzene (DNCB), 2-phenyl-4-ethoxymethylene-5-oxazolone (OXA), and toluene 2,4-diisocyanate (TDI) as a strong contact sensitizer, a skin contact sensitizer and a skin or respiratory sensitizer, respectively, and the irritants, croton oil (CRO) and nonanoic acid (NA). All test chemicals were freshly prepared in vehicle (4:1 v/v acetone/olive oil, AOO).

2.3. Treatments

Two kinds of experiments were performed using different animals for each experiment: single dose experiment for cytokine immunoassay and multiple doses experiment for the real-time RT-PCR analysis of cytokines and GzmB. In single dose experiment, four mice per group were treated with one dose known to evoke clearly a sensitizing or irritating response in mice; 1% (w/v) DNCB (Van Och et al., 2002), 1% (w/v) OXA (He et al., 2001), 1.5% (w/v) TDI (He et al., 2001), 1% (v/v) CRO (Homey et