
A noninvasive method for quantifying and distinguishing inflammatory skin reactions

Vera B. Morhenn, MD,^a En-Yuh Chang, PhD,^b and Lawrence A. Rheins, PhD^a
San Diego, California

Background: The ribonuclease protection assay (RPA) represents a technology that allows detection of small amounts of intact RNA. Recent progress in understanding cytokine networks in the skin suggests that measurements of cytokine mRNA levels could provide a method to distinguish various reactions such as irritant contact dermatitis and allergic contact dermatitis that can occur in the skin.

Objective: We attempted to differentiate and quantitate irritant and immunologic skin reactions by measuring mRNA levels.

Methods: We have used the technique of tape stripping human skin to remove superficial cell layers and have extracted RNA from these skin samples. This RNA was used for RPA analysis.

Results: By means of RPA analysis, we have demonstrated distinct cytokine profiles that appear to discriminate, for example, irritant from immunologic skin reactions.

Conclusion: We have shown that multiple cytokine mRNA levels can be defined in these RNA samples obtained from the skin. This approach assesses not only the cytokine gene profiles, but at the same time may quantify the severity of common irritant versus allergic skin reactions. (*J Am Acad Dermatol* 1999;41:687-92.)

The skin is the most accessible organ of the body and is the site of many endogenous inflammatory reactions as well as those that are induced by exogenous agents. Both allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) can cause blistering and erythema in the skin. Clinically, these reactions are usually indistinguishable.^{1,2} This can make determining the exact origin of a dermatitis difficult.

The outermost layer of the integument, the epidermis, consists of several cell types. The most prevalent cell is the keratinocyte in various stages of differentiation. This cell is a veritable factory of cytokines including interleukin-1 (IL-1), IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, and granulocyte-macrophage/colony-stimulating factor (GM-CSF).³ Interferon gamma (IFN- γ) can induce HLA-DR antigen expression by the keratinocyte, and in contact dermatitis the keratinocyte may express this anti-

gen.^{4,5} In an allergic reaction, the keratinocytes rapidly synthesize the mRNA for IFN- γ .⁶ Furthermore, in these cells, IFN- γ can induce the mRNA for the inducible isoform of nitric oxide synthase (iNOS), which in turn can produce nitric oxide, a potent vasodilator.^{7,8} Above the basal cell layer resides the Langerhans cell, an immune competent cell of bone marrow origin.⁹ The Langerhans cells have features of macrophages as well as T cells and are thought to be responsible for initiating the series of events that lead to immune reactions in the skin such as those manifested by contact dermatitis.¹⁰

The ribonuclease protection assay (RPA) can identify and quantitate small amounts of mRNA.¹¹ The mRNA levels in a cell in most cases reflect the levels of the corresponding proteins. Thus a measurement of mRNA should provide an objective monitor of events such as inflammatory reactions occurring in the skin. To differentiate and quantitate ACD and ICD reactions using a relatively noninvasive technique, the following experiments were performed.

MATERIALS AND METHODS

Induction of erythematous reactions on the skin

The ICD was induced by applying 0.5% sodium lauryl sulfate (SLS, Fisher Scientific, San Diego, Calif) in distilled

From the California Skin Research Institute, San Diego,^a and the La Jolla Institute for Allergy and Immunology.^b

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Reprint requests: Vera B. Morhenn, MD, Clinical Associate Professor, University of California at San Diego, California Skin Research Institute, 15222-B Avenue of Science, San Diego, CA 92128.

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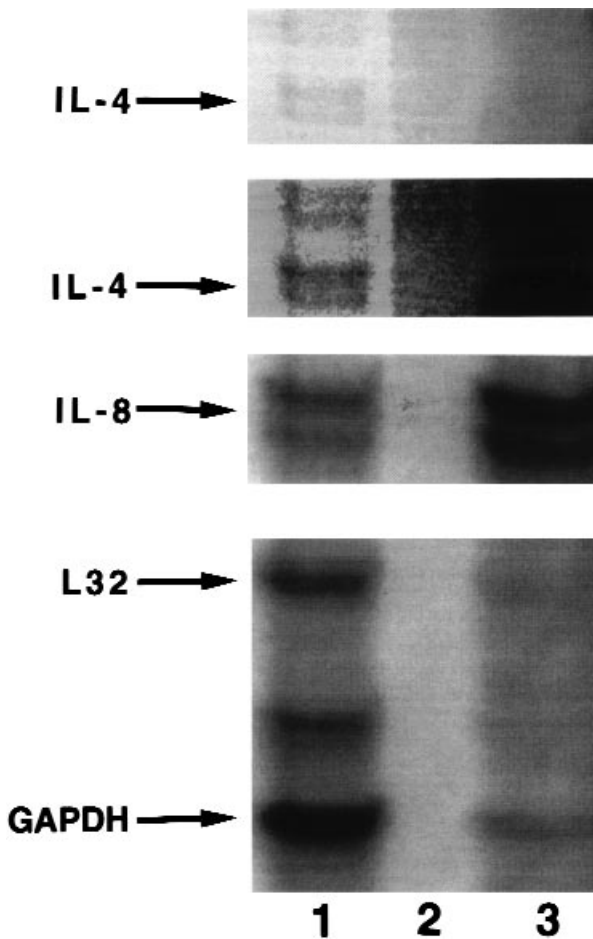


Fig 1. Four RNA probes (IL-4, IL-8, L32, GAPDH) were used for hybridization to RNA samples from 1 individual. Cells obtained by tape stripping (12 times) skin sites on upper arms of this individual (same as subject 2, Fig 2) were analyzed by RPA. Lane 1 is RNA isolated from squarate-induced erythematous area of skin, read clinically as 2+ erythema. Lane 3 is RNA isolated from 0.5% SLS-induced erythematous site (scored 2+). Both lanes clearly demonstrate bands for IL-8. Upper panel is short exposure of phosphor screen. (RNA for housekeeping genes in lane 1 is more intense, therefore amount of RNA used is larger than amount used in lane 3.) Lane 2 is sample obtained from noninflamed, normal appearing skin of same individual, which contains very little RNA. Second panel from top shows longer exposure of phosphor screen; band for cytokine IL-4 seen only in lane 1 that contains RNA from ACD reaction. Bottom two panels show higher IL-8 level in lane 3 than in lane 1, despite weaker IL-4, L-32, and GAPDH levels.

water for 72 hours. At this point, the erythema was graded according to a standard scoring scale and tape stripping was performed (see below).¹² Dibutyl squarate (2%) in acetone was applied for 48 hours under occlusion to the upper arm to induce the ACD reactions. After about 14 days the dibutyl squarate (2%) was reapplied to elicit the

immune-mediated reaction. The scoring of the erythema as well as tape stripping was performed 48 hours later.

Tape stripping

We removed skin cells, using D-SQUAME tape (CuDerm, Dallas, Tex) to strip the skin's cell layers.¹³ The skin was stripped with the tape up to 23 times. The tape was placed in an RNase-free Eppendorf tube containing 0.5 mL of a commercially available RNA extraction mixture, Tri Reagent (see Reagents). The RNA was extracted by vortexing and centrifuging the tape in the Eppendorf tube (see RNase protection assay). RNA from tapes used at the same skin site was extracted with and pooled in 0.5 mL Tri Reagent. For the later experiment, the first 3 strips of tape were discarded after stripping to expose the inner layers of skin for more effective RNA extraction. Only the next 20 tape strips were processed for the RPA analysis. This entire procedure was performed in less than 90 minutes.

Reagents

Tri Reagent for total cellular RNA isolation was purchased from Molecular Research Center, Inc (Cincinnati, Ohio); RiboQuant multi-probe RNase protection assay system and hCK1 probe sets were purchased from PharMingen, Inc (San Diego).

RNase protection assay

After each tape stripping, RNA was immediately extracted by vigorously vortexing the tape in 0.5 mL Tri Reagent. Yeast transfer RNA (4 μ g) was then added as carrier RNA before the total RNA was isolated and purified according to the manufacturer's instruction. The total RNA so isolated from each sample was used directly for the RNase protection assay by means of the RiboQuant multi-probe RNase protection assay system without prior determination of the amount of RNA by OD measurement. Assays were performed according to the manufacturer's protocol. Undigested probes were run parallel with samples on standard acrylamide sequencing gels and used to identify digested cytokine messages. Gels containing digested RNA bands were first exposed to a Phosphor Screen (Molecular Dynamics, Inc, Sunnyvale, Calif). The exposed screen was then scanned with a phosphor imager Storm 860 (Molecular Dynamics, Inc). Intensities of bands in each sample were analyzed with the software ImageQuant (Molecular Dynamics, Inc).

RESULTS

Cytokines identified by RPA

We initially used 4 RNA probes (IL-4, IL-8, L32, GAPDH) for hybridization to RNA samples obtained from 1 individual. Fig 1 shows the cytokines identified by RPA analysis of the cells obtained by tape stripping (12 times) skin sites on the upper arms of this individual (the same individual as subject 2 in Fig 2). Demonstrated in lane 1 is the RNA isolated from an erythematous area of skin, read clinically as 3+ erythema, that was induced by squarate. Lane 3

shows the RNA isolated from an erythematous site (scored 2+) induced by 0.5% SLS. Both lanes clearly demonstrate the bands for IL-8. (Because the RNA for the housekeeping genes in lane 1 is more intense, the amount of RNA used in this lane is larger than the amount of RNA used in lane 3.) Lane 2 represents a sample that contains very little RNA, obtained from noninflamed, normal-appearing skin of the same individual.

After longer exposure of the phosphor screen, the band for the cytokine IL-4 can be seen only in lane 1, the lane that contains the RNA from an ACD reaction (Fig 1, second panel from top). It can be seen clearly that lane 3 contains a higher IL-8 level than lane 1, despite its weaker IL-4, L32, and GAPDH levels (Fig 1, bottom 2 panels). Thus the cytokine pattern in the ACD reaction differed from the ICD reaction.

In 3 of the 5 subjects tested in a subsequent experiment, the cytokine IL-4 again was clearly visible only in the RNA obtained from skin areas that had acquired an allergic reaction (lanes 8, 11, and 13 in Fig 2). By contrast, IL-4 was much weaker in any of the SLS-treated areas of skin or in normal skin samples obtained from the same subjects. Lanes 5 and 12 contain RNA samples from skin areas that demonstrated vesicles. In these areas, tape stripping was impeded by the serous exudate from the blisters. In our experience, this often resulted in smeared lanes containing excessive IL-8 message in the RPA analysis. Furthermore, in 4 of 5 subjects (subjects 2, 3, 4, and 5 in Fig 2), IL-8 was present in the RNA obtained from all of the erythematous areas of skin, whether the erythema was induced by an irritant reaction or an allergic one, but not in the RNA obtained from normal skin. Because samples obtained from the other individual (subject 1 in Fig 2) contained a relatively small amount of RNA, the result for this subject was inconclusive.

The RNA for the cytokine IL-13, a protein secreted by activated T cells, was present in 3 (lanes 8, 11, and 13 in Fig 2) of the 4 erythematous areas of skin (lanes 5, 8, 11, and 13 in Fig 2) in which inflammation had been induced by the allergen and for which the lane could be visualized (Fig 2).¹⁴ As mentioned above, 2 of the lanes (lanes 5 and 12; subjects 2 and 5 in Fig 2) contained smudges, making assessment of the RNA for IL-13 difficult for these 2 subjects.

Faint bands could be seen in the approximate areas expected to contain the RNA with the molecular weight for IFN- γ from 2 (lanes 8 and 11 in Fig 2) of the 5 squarate-treated skin samples. The band for the putative RNA for IFN- γ was seen in 2 of the lanes that also demonstrated the RNA for IL-4.

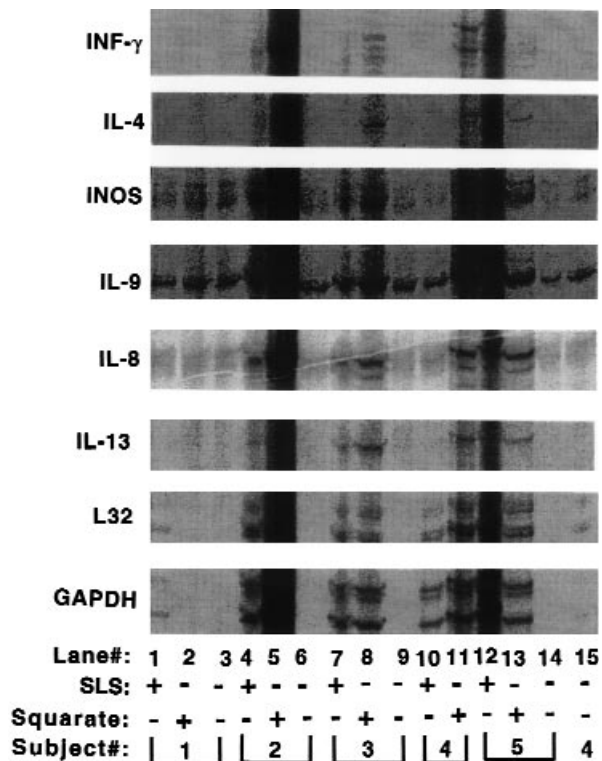


Fig 2. Results for RPA performed with RNA obtained by tape stripping 3 areas of upper arms of 5 individuals. Riboprobes for 6 RNAs (IL-4, -8, -9, -13, IFN- γ , iNOS) plus 2 housekeeping genes were included. At bottom, + indicates skin harvested from subjects' arm had been treated either with SLS (second row at bottom) or squarate (third row at bottom). Bottommost row indicates, in brackets, various treatments for a single subject.

IL-9 (a multifunctional cytokine) and iNOS were detected in all 13 samples that could be visualized in this experiment (Fig 2).¹⁵

Clinical scores

The clinical quantification of the erythema visualized in the various skin reactions is documented in Table I. IL-4 could be seen in ACD skin reactions graded 2+. IL-8 could be seen in every ICD reaction (1+ to 3+) regardless of the degree of erythema.

Comparison of the skin reactions by means of RPA

To compare the degree of skin inflammation by means of RPA, the amount of IL-4, IL-8, and iNOS present in each individual lane in Fig 2 was first normalized to the amount of the corresponding housekeeping gene, GAPDH, and the resulting values for ACD reaction were then divided by their corresponding ICD values. As mentioned before, samples from subject 1 contained very little RNA, and the

Table I. Correlation of the degree of erythema in the skin sites' reaction and the presence or absence of the 2 cytokines IL-4 and IL-8

Subject No.	Degree of skin reaction		Cytokine pattern	
	ACD	ICD	ACD	ICD
2a*	2+	2+	IL-4+/IL-8+	IL-4-/IL-8+
1	0	0	IL-4 ND/IL-8 ND*	IL-4 ND/IL-8 ND
2b†	3+	0‡	IL-4?§/IL-8?§	IL-4-/IL-8+
3	2+	1+	IL-4+/IL-8+	IL-4-/IL-8+
4	2+	±1+	IL-4+/IL-8+	IL-4 ND/IL-8±
5	2+	3+	IL-4+/IL-8+	IL-4?§/IL-8+

Where all lanes could be visualized, IL-4 could be seen only in RNA harvested from skin where an ACD reaction could be visualized.

ND, Not detected.

*Subject shown in Fig 1. This is the same subject as subject 2, but tested on a different day.

†Subject shown in Fig 2.

‡Some pruritus.

§Smear lanes.

Table II. Ratios of normalized cytokine and iNOS messenger RNA levels between ACD and ICD reactions

Subject No.	Cytokine or protein	ACD	ICD
3	IL-4	>>0	0*
	IL-8	2.5	1
	iNOS	1.2	1
4	IL-4	>>0	0*
	IL-8	2.1	1
	iNOS	3.7	1
5	IL-4	>>0	0*
	iNOS	3.7	1

Cytokine as well as iNOS mRNA levels from each sample were first quantitated by means of the software ImageQuant. Readouts (with background subtracted) for cytokines in each sample were then normalized to corresponding GAPDH readout. The ratio for each pair of normalized cytokine (or iNOS) levels for each subject between ACD and ICD reactions were taken and reported. ICD reactions never contained detectable amounts of IL-4; therefore, ratios for this cytokine were not calculated.

*The level of IL-4 message was not detectable in ICD reactions.

ACD sample from subject 2 contained extensive smudge (ie, lane 5 in Fig 2). These 2 individuals were thus excluded from this analysis. As shown in Table II, among the 3 subjects (3, 4, and 5) analyzed, a nice correlation exists between the RNA levels and the severity of the reactions in that the samples from the strong skin reactions were also the ones that demonstrated large relative amounts of IL-8 for the ACD reaction. Furthermore, ACD reactions in general

showed larger amounts of IL-4, IL-8, and iNOS when compared with ICD reactions.

DISCUSSION

These results demonstrate that skin RNA can be obtained from tape strips without significant degradation as indicated by the ability to detect even low abundant RNA species such as IFN- γ and iNOS (Fig 2). These mRNAs potentially can be characterized in a variety of ways including assessing the presence of cytokine mRNA for various interleukins as was done in this report. The exact species of mRNA probes used in these experiments was determined in part by the necessity for using probes of different molecular weights in one and the same lane. The probes for the control housekeeping genes also were included in these experiments and provided a means of quantitating the relative amount of mRNA present in each sample. Because the skin is a rich source of RNase that can quickly degrade RNA released from damaged epidermal cells, the collection and extraction techniques described here take into account the risk of digesting epidermal RNA. By means of this technique, we have isolated RNA from tape-stripped skin in collaboration with 3 different investigators using different laboratory settings (not shown).

We chose the technique of RPA to characterize the reactions described herein because this method has certain advantages over reverse transcriptase-polymerase chain reaction (RT-PCR) or in situ hybridization. The technique of RPA is both extremely sensitive (allowing analysis of small amounts of RNA collected noninvasively) and inherently quantitative (unlike RT-PCR or in situ hybridization).

In a recent study, subjects with known ACD were challenged with allergen, and increased numbers of epidermal keratinocytes were shown to express IFN- γ by *in situ* hybridization.¹⁶ By contrast, no consistent changes in IL-1 α or IL-8 expression were observed. Using RT-PCR and shaved skin biopsy specimens from human subjects, Grängsjö et al¹⁷ detected increased IL-6 but not GM-CSF mRNA expression after exposure to the irritant nonamoxic acid; whereas SLS increased GM-CSF but not IL-6. IL-4 was found in ACD reactions. It is clear from these data that epidermal cells can synthesize cytokines, and that both ACD and ICD reactions can upregulate discrete sets of cytokines.

The presence of IL-4 and IL-13 in squaric acid-treated skin samples demonstrated here strongly suggests that these cytokines were induced by a mechanism that is specific to the allergic reaction in the skin from which these samples were obtained. However, we have not totally ruled out that the intensity of the clinical reaction also may have influenced the appearance of the IL-4/IL-13 cytokine pattern, because, on the whole, the ACD reactions were stronger than the ICD reactions, although these two cytokines have not been demonstrated in the ICD reactions by means of other techniques.¹⁸⁻²⁰ We plan to confirm our findings by performing biopsies of the two types of skin reactions, that is, ACD and ICD. We will then characterize the protein patterns on the biopsy specimens by means of immunohistochemistry and compare these patterns to the results from the RPA performed in parallel on the same individual(s).

In 3 of the subjects tested (3, 4, and 5), the degrees of inflammation in the erythematous skin sites could be correlated with the IL-4 and IL-8 RNA levels when normalized to the corresponding housekeeping gene levels (Table II). Thus, subject 4, who demonstrated a 2+ reaction in his ACD site and only a ± 1 reaction in his ICD site, also showed an approximately 2-fold difference in the IL-8/GAPDH ratios when comparing the ACD and ICD reactions by means of the RPA method.

The process of tape stripping itself has been shown not to affect the skin cytokine profile during the first few hours after the procedure is done.²¹ Furthermore, during the early hours after stripping no inflammatory cells migrate from the circulation into the dermis or epidermis.

Dermatitis may result from a broad array of external agents applied to the skin. Classes of dermatitis may include irritant, allergic, photoallergic, and phototoxic mechanisms.²² Clinically, these reactions are virtually identical to the appearance of an eczematous process typified by erythema, edema, and vesic-

ulation. Categorizing the mechanism(s) of the allergic reaction to various cosmetic products, for example, should be of great importance for consumers as well as manufacturers. These reactions usually stem from the immunologic consequences of an allergic or immune response that eventually lead to increasingly severe inflammation of the skin with re-exposure after initial sensitization. Recurrent skin eruptions will be likely to continue in these instances even after the offending component of the product has been reduced quantitatively. In contrast, irritant reactions in the skin may be reduced or eliminated by product reformulation.

The approach described here appears to distinguish between two types of reactions (ie, ICD and ACD) in a matter of a few days rather than several weeks, which is the time needed to perform the current standard clinical techniques.²³ Although other methods for assessing the effects of phototoxic agents, as well as ICD and ACD, have been published recently, none of these were performed on human skin *in vivo*.²⁴⁻²⁶ To optimize the technique, we recently have performed experiments to determine the optimal number of tape strips that have to be removed from the skin and subsequently harvested (not shown). These results suggest that the first 10 tape strips harvested (that probably contain material from mainly the nonnucleated stratum corneum) do not need to be extracted with Tri Reagent but simply can be discarded.

The noninvasive tape stripping technique described here could be applied to a number of other areas such as isolating small fragments of DNA for assessing the protection against photodamage. Finally, the cytokine patterns induced by a variety of other skin, systemic, or neurologic diseases also could be assessed with this technique.

Since this manuscript was submitted for publication, an article appeared describing the characterization of cytokines in antigen activated skin of patients with atopic dermatitis by combining the techniques of skin scraping and RT-PCR.²⁷

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