Three Predictive Tests Using Mice for the Identification of Contact Sensitizer

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Abstract

Predictive tests for the identification of contact sensitizing chemicals have been developed. We measured the sensitization potential with three predictive tests, the *in vitro* and the *in vivo* Local Lymph Node Assay(LLNA), ELISA to detect interferon-gamma(IFN-γ) from supernatant and flow cytometry to detect change of cell surface proteins, using draining lymph nodes of mice.

BALB/c mice were exposed to various chemicals or vehicles on the ears daily for 3 consecutive days in all experiments. With some exceptions of propyl paraben, neomycin sulfate, the *in vivo* LLNA was able to detect the sensitizing capacity of test chemicals and was more sensitive than the *in vitro* LLNA for chemicals used in the present study. In another experiment, contact sensitivity was assessed by the ELISA to detect IFN-γ from the supernatants of the cultured LNCs after sensitization with chemicals. There was a good correlation between the LLNA and the IFN-γ production for test chemicals. We also examined the change of cell surface proteins on LNCs after sensitization by flow cytometry for some cell adhesion molecules(ICAM-1, E-cadherine, B7 molecule), T cell markers(CD3, CD4, CD8, Tαβ, Tγδ) and B cell markers(LR1, CD45R, I-A^d). The number of ICAM-1 positive cells and B cells in LNCs were increased after sensitization with DNCB, TNCB, isoeugenol and 25%, 50% cinnamic aldehyde compared with that of vehicle as a control.

In conclusion, the *in vivo* LLNA could provide more sensitive screening test for moderate to strong sensitizers and some weak sensitizers including cosmetic raw materials than the *in vitro* LLNA. The production of IFN-γ by allergen-activated LNCs might be a valuable indicators without radioisotopes for the identification of contact allergens. Detection of allergens by testing the increase of ICAM-1 positive cells and B cells in LNCs by flow cytometry might be used as a test method to detect allergens.

Introduction

Allergic contact dermatitis is a delayed-type hypersensitivity(DTH) reaction in the skin characterized by a tissue damaging inflammatory response. The skin sensitizing potential of chemicals is assessed currently by using a variety of guinea pig test methods. Various guinea-pig test methods are performed by measuring cutaneous erythema and/or oedema in suitably induced and challenged animals(1,2,3). The limitations of these methods are that they rely on subjective skin reaction grades, thereby producing limited quantitative data. In addition, they are time consuming and require relatively large number of animals. In recent years, attention has focused on the mouse as an alternative model for predictive tests of contact allergy(4). The most thoroughly studied method is the mouse ear swelling test(5) and the LLNA(6). The use of the LLNA has been recently developed as more quantifiable method for predictive testing. This test determines sensitization potential by assessment of proliferative responses in lymph nodes draining the site of chemical application. LNCs proliferation activity was assessed by *in vitro,* following culture of draining LNCs with ^{3}H-methylthymidine(^{3}[H]TdR) (*in vitro* LLNA)(7). Later, mice were injected intravenously with ^{3}[H]TdR and the amount of ^{3}[H]TdR incorporation in the entire draining lymph node were measured (*in vivo* LLNA)(8). The *in vivo* LLNA has been the subject of comparisons with guinea pig test methods(9) and of extensive interlaboratory validation studies(10). On the other hand, the *in vitro* LLNA also has been studied and modified to improve the sensitivity in many research groups(11). Therefore we compared the sensitivity of the *in vivo* with the *in vitro* LLNA for 12 test chemicals. The LLNA could provide a rapid, objective, quantitative and cost-effective method for screening chemicals that possess moderate or strong sensitizing potential sensitisers, additionally for screening many weak sensitizers. In spite of these many advantages of the LLNA, this test has a disadvantage, using radioisotope. To overcome this disadvantage, we developed the modified method without using radioisotope which measured molecular endpoints instead of cellular endpoints to detect contact sensitizers. We investigated the production of INF-γ from supernatant and the change of surface proteins by draining LNCs. IFN-g is the most important mediator of DTH and is produced by Th1 CD4+ helper T cells and nearly all CD8+ T cells. IFN-γ stimulates class I, II MHC molecule expression and is a potent activator of mononuclear phagocytes and acts directly on T and B lymphocytes to promote their differentiation and activate vascular endothelial cells(12). DTH is cell-mediated immunity, which is directed at or near cells that bear foreign antigens on their surface. During the DTH reaction, the amount of surface proteins was changed essentially, therefore we tested ICAM-1, E-cadherine, B7 molecule as cell adhesion markers and CD3, CD4, CD8, T$\alpha\beta$, T$\gamma\delta$ as T cell markers and LR1, CD45R, I-A^{d} as B cell markers.

In this study, we examined the allergenicity of 29 chemicals using the *in vitro* LLNA and compared between the *in vivo* and the *in vitro* LLNA. We also investigated on IFN-γ production and the change of surface proteins using draining lymph node of mice to predict the allergenecity potential of these chemicals without using radioisotope.

Experimental

Materials

Trimellitic anhydride(TMA), Dicyclohexylmethane-4,4'-diisocyanate(HMDI), Diphenylmethane-4,4'-diisocyanate(MDI), Cobalt chloride, Propyl gallate, Cinnamic aldehyde were purchased from Aldrich. Kethon CG was purchased from Rohm & Haas. 2,4,6-Trinitrochlorobenzene(TNCB) was purchased from Tokyo Kasei chemical. Laurylmethicone copolyol was purchased from Dow Corning and Oleth-15 was purchased from Nihon. Dimethylformamide(DMF) was purchased from Junsei. 4-Ethoxymethylene-2-phenyloxazol-5-one(Oxazolone), Nickel sulphate, Propyl paraben, Imidazolidinyl urea, neomycin sulfate, 2,4-Dinitrochlorobenzene(DNCB), Formaldehyde, Potassium dichromate, Glycolic acid, 2-Methoxy-4-propenylphenol(Isoeugenol), Eugenol, Sodium lauryl sulfate, Dimethylsulfoxide, Olive oil were purchased from Sigma. Test chemicals were dissolved in 4:1 acetone:olive oil(AOO) or DMF or DMSO.

Rat anti-mouse cadherin mAb ECCD-2 were provided by M.Takeichi(Kyoto University). Rat anti-Thy 1.2, rat anti-B7-1 and B7-2, rat anti CD-45R(B220), rat anti-B cell antigen(LR-1) and fluorescein isothiocyanate(FITC) conjugated rat anti-I-A^d were purchased from PharMingen(SanDiego, CA). Rat anti-CD3 was purchased from BioSource (Camarillo, CA) and FITC conjugated hamster anti-mouse γδ T cell receptor(TCR) and anti-mouse αβ TCR from Boehringer Manheim Corp(Indianapolis,IN). Hybridoma secreting anti-mouse ICAM-1(YN1/1.7.4) mAb was obtained from ATCC(Rockville, MD). FITC-conjugated $F(ab)^2$ fragment of goat anti-mouse was purchased from Tago(Camaeillo, CA).

Animals

Young adult(6-8 weeks) BALB/c strain mice were used throughout these studies.

In vitro *LLNA*

Twenty five microliter of test chemical solution or vehicle were applied to both ears of mice daily for 3 or 5 days. On next day after final application, draining (auricular)lymph nodes were excised and pooled for each experimental group. A single cell suspension of lymph node cells(LNCs) was prepared by mechanical disaggregation through sterile 200-mesh gauze. The concentration of LNCs suspension was adjusted to 1x107 cells/ml in RPMI-FCS. LNCs suspensions were seeded into 96-well plates and cultured for

24h at 37°C in a humidified atmosphere of 5% CO_2 in air with 2 mCi of 3H-TdR(Amersham International, Amersham, U.K.). The culture was terminated by automated cell harvesting and ^{3}H-TdR incorporation was determined by β-scintillation counting.

In vivo *LLNA*

Induction procedure was same as that of the *in vitro* LLNA. Five days following the initiation of treatment, all mice were intravenous injected via the tail vein with 250μl of phosphate-buffered saline(PBS) containing 20mCi of [$_3$H]-TdR. Five hours later, mice were sacrificed and the draining auricular lymph nodes were excised and pooled for each experimental group. A single cell suspension of LNCs was prepared by mechanical disaggregation. Pooled LNCs were washed twice with an excess amount of PBS and precipitated with 5% trichloroacetic acid(TCA) at 4°C. Incorporation of $_3$HTdR was measured by β-scintillation counting.

Determination of IFN-γ production

LNCs proliferative response and IFN-γ production were measured in parallel cultures. LNCs suspensions were seeded into 96-well tissue culture plates and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Supernatants were collected after incubation, centrifuged at 7000g for 3 min and stored at -70°C. IFN-γ production was assayed by Enzyme-linked Immunosorbent Assay (ELISA). Briefly, supernatant obtained after centrifugation of LNCs was added to a 96-well tissue culture plate pre-coated with anti-IFN-γ antibody and incubated for 2 hr at room temperature. The plate was washed with buffer solution, then the plates were incubated for 2 hr at room temperature with polyclonal goat anti-murine IFN-γ, and for a further 1 hr at room temperature with donkey anti-goat-peroxidase. Enzyme substrate was added and incubated for 10 min. Optical density at 450 nm was measured. Results were expressed as IFN-γ concentration in pg/ml

Preparation of Murine draining LNCs and Flow cytometry

Mice were sacrificed by cervical dislocation at 5th day after application of chemicals. Immediately after sacrificing the mice, draining LNCs was prepared by mechanical disaggregation. The LNCs were used for flow cytometry. In single color immunofluorescenece experiments, the LNCs was incubated with optimal concentrations of monoclonal antibodies to the surface proteins of LNCs or with purified isotypematched control mAb and FITC-(Fab')^2 fragments of affinity-purified goat anti-rat IgG, and then analyzed for surface Ag expression by flow cytometry(FACScan, Becton Dickinson)

Results and Discussion

Validation of the in vitro *LLNA*

The *in vitro* LLNA were validated with 29 well-characterized sensitizing chemicals of varying potency and several cosmetic raw materials. The results of the *in vitro* LLNA are summarized in Table 2. Fold of increase are shown as the ratio of proliferation in the test group to that in the vehicle control group. A chemical is regarded as a positive(sensitizer) in this assay if treatment of at least one concentration of chemical showed a ratio of equal to or greater than 3. When the number of applications were increased from three to five, both the amount of ^{3}[H]TdR incorporation and fold of increase were increased(data not shown). The repeated application(increase the number of test chemical application) was effective for increasing the sensitivity. The potent sensitizing chemicals(such as oxazolone, TMA, dinitrochlorobenzene) resulted in remarkable increases in LNCs proliferation. However, weak sensitizer(geraniol, neomycin sulfate) failed to cause changes in LNCs proliferation. Propyl paraben, which was classified as a non-sensitizer in Guinea Pig Maximization Test also showed negative response. Nickel sulphate showed equivocal response, sometimes it showed positive results and sometimes negative. It may be due to its poor solubility to vehicle(DMSO). Irritants(SLS, Triton x-100) also induced increase LNCs proliferation. Glycolic acid failed to elicit positive response although it caused necrosis on mouse ear. It was considered that glycolic acid was an irritant rather than allergen in this study. Other chemicals(laurylmethicone copolyol, oleth-15) which were used as emulsifiers yielded greater than 3 fold increase in their proliferation response.

Comparison the in vitro *with the* in vivo *LLNA*

We compared the results of the *in vivo* LLNA and the *in vitro* LLNA for 12 chemicals. The data recorded in Table 2 illustrated that the *in vivo* LLNA appeared to be more sensitive than the *in vitro* LLNA. The sensitizing chemicals(Geraniol, dimidazolidinyl urea) induced positive response in the *in vivo* LLNA, while these chemicals cause negative responses in the *in vitro* LLNA. The fold of increase in the *in vivo* LLNA was higher than that of the *in vitro* LLNA for most chemicals. This difference in sensitivity might be due to the decrease of the LNCs activity influenced by environmental change in the *in vitro* system. However neomycin sulfate, propyl paraben failed to induce positive response in both the *in vivo* and the *in vitro* LLNA. This result suggest that the *in vivo* LLNA could provide a sensitive screening test for moderate to strong sensitizers and some weak sensitizers.

Interferon-γ production

We evaluated the potential of molecular (IFN-g production), instead of cellular(lymphocyte proliferation) end-points for determining the allergenecity of the chemicals. Figure 1 and 2 represents the effect of cell culture time on IFN-γ production from LNCs. The LNCs prepared from mice exposed to 1% DNCB or 1% Oxazolone were cultured for various incubation times either 3 to 48 hr or 6 to 120 hr. The kinetics of IFN-γ production of DNCB in these experiments were different from those observed for OXAZ. DNCB elicited maximal level of IFN-γ production at 72 hr incubation and levels maintained for up to 120 hr, while OXAZ reached a plateau at 24 hr.(Figure 3,4) Different kinetics of cytokine expression might be due to different potential of chemical allergenecity. Figure 3 and 4 showed the effect of chemical concentration of oxazolone and DNCB. All inducing concentrations of oxazolone(0.25,0.5,1%) and DNCB(0.5,1,2%) produced increased levels of IFN-γ production, compared with control prepared from vehicle-treated mice. Considering the IFN-g production patterns of two chemicals, the IFN-g production during 24 hr culture was selected as a marker for predictive of contact allergens. The result of IFN-γ production, the *in vivo* and the *in vitro* LLNA induced by test chemicals are summarized in Table 3. The potent contact sensitizing chemicals DNCB, OXAZ, cinnamic aldehyde, isoeugenol induced high levels of IFN-γ production. Eugenol, moderated compounds induced comparatively low levels of IFN-γ production. However kathon CG which showed positive response in the LLNA did not induce measurable IFN-g production. The nonsensitizing chemicals, ethanol, AOO, DMSO failed to provoke detectable levels of IFN-γ production. We didn't have any criterion to represent the positive response because we haven't obtained enough information about moderate sensitizers yet. Taken together, these data suggest that the production of IFN-γ by allergen-activated LNCs could serve as a selective marker for contact allergen. Measurement of IFN-γ production has a advantage which does not require the use of radioisotope, although it is less sensitive than mesurment of the thymidine incorporation in the LLNA.

Flow cytometry assay

Mice were treated on the ears for three consecutive days and phenotypic analysis of LNCs was made at 48 hr following the final treatment. We examined the change of some cell adhesion molecules(ICAM-1, E-cadherine, B7 molecule), T cell markers(CD3, CD4, CD8, Tαβ, Tγδ) and B cell markers(LR1, CD45R, I-Ad). The number of ICAM-1 positive cells and B cells in LNCs were increased after sensitization with DNCB, TNCB, isoeugenol and 25%, 50% cinnamic aldehyde compared to that of vehicle. (Figure 7,8,9) The upregulation of ICAM-1 may be related to the facilitation lymphocyte movement into epidermis in response to contact allergen. Other surface proteins did not provide any significant meaning. Although this approach needs further investigation on a wide range of chemicals, it could inform us of additional characteristics of test chemicals with respect to the immunobiology.

Conclusion

In the present studies, it was concluded that the *in vivo* LLNA was more sensitive method compared with the *in vitro* LLNA, and could be a predictive method to screen the contact allergenicity of cosmetic raw materials. The IFN-γ assay might be an alternative to radioisotope-dependent proliferation testing for moderate to strong allergens. It also indicated that detection of the change of surface protein (Ia^d, LR-1, ICAM-1 expression) could aid in identifying sensitization potential of chemicals.

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Table1. Validation of the *in vitro* LLNA : An examination of 29 chemicals

Chemical	Test concentration (%)	Vehicle	No. Of application time	Mean LNCs proliferation (cpm)	Fold-increase	Clasification
OXAZ	0.125	AOO	3	102705	9.1	+
	0.25	AOO	3	199462	17.7	+
	0.5	AOO	3	207191	18.4	+
	1	AOO	3	221404	19.6	+
TMA	1	AOO	3	48564	16.8	+
HMDI	0.25	AOO	3	53037	18.4	+
MDI	1	AOO	3	74844	25.9	+
TNCB	1	AOO	3	36479	12.6	+
DNCB	0.5	AOO	3	19873	7.8	+
	1	AOO	3	20601	8.1	+
	2	AOO	3	16052	6.3	+
Formaldehyde	25	AOO	3	22656	7.9	+
Cinnamic aldehyde	10	AOO	5	14991	4.4	+
Isoeugenol	10	AOO	5	14817	4.7	+
Eugenol	50	AOO	5	16007	4.4	+
Geraniol	75	AOO	3	3459	1.2	-
Kathon CG	1	DMF	3	13540	3.5	+
Germall II	50	DMSO	3	2400	0.8	-
Neomycin sulfate	30	DMSO	3	1863	0.7	-
Nickel sulfate	30	DMSO	3	12601	3.1	+/-
Propyl paraben	50	AOO	3	2555	0.8	-
Cobalt chloride	10	DMSO	3	42209	4.3	+
Propyl gallate	50	AOO	5	23257	6.6	+
Potassium dichromate	50	AOO	3	35318	10.0	+
Laurylmethicone copolyol	50	AOO	5	12883	3.7	+
Oleth-15	50	AOO	3	22742	6.4	+
Glycolic acid	25	DMF	5	2166	0.6	-
SLS	25	DMSO	3	22360	2.7	-
Triton x-100	25	AOO	3	17955	5.4	+
AOO			3	2887		
MEK			3	4019		
DMF			3	4139		

Ethanol	30	Water	3	3115
DMSO			3	7814

Fold of increase are shown as the ratio of proliferation in the test group to that in the vehicle control group.

+ = positive; - = negative; +/- = equivocal.

AOO = acetone-olive oil (4;1, v/v)

Table 2. The *in vivo* and *in vitro* LLNA ; A comparative analysis

	Concentration	Fold-Increase	
Test Chemicals	(%)	*in vitro* LLNA	*in vivo* LLNA
TNCB	1	+(12.6)	+(21.7)
HMDI	0.25	+(18.4)	+(31.6)
DNCB	1	+(8.1)	+(10.9)
Cinnamic aldehyde	10	+(4.4)	+(3.2)
Isoeugenol	20	+(4.7)	+(5.3)
Eugenol	50	+(4.4)	+(8.0)
Geraniol	75	-(1.2)	+(7.4)
Imidazolidinyl urea	50	-(1.1)	+(3.3)
Nickel sulfate	30	+/-	+/-
Propyl paraben	50	-(0.7)	-(1.3)
Glycolic acid	20	-(1.37)	-(2.0)
Neomycin sulfate	30	-(0.7)	-(2.7)

Fold of increase are shown as the ratio of proliferation in the test group to that in the vehicle control group.

+ = positive; - = negative; +/- = equivocal.

AOO = acetone-olive oil (4;1, v/v)

Table 3. Result of LLNA and production of IFN-γ (pg/ml) by draining LNCs after contact with several kinds of allergens and vehicles

	IFN-γ production pg/ml	Classification *in vitro* LLNA	*in vivo* LLNA
Oxaz 0.5%	25103	+	+
DNCB 0.5%	12329	+	+
Cinnamic aldehyde 10%	3539	+	+
Isoeugenol 10%	1755	+	+
Eugenol 50%	472	+	+
Imidazolidinyl urea 50%	-	-	+
Neomycin sulfate 30%	-	-	-
Nickel sulfate 30%	-	+/-	+/-
Kathon CG	-	+	nd
Propyl paraben 50%	-	-	-
Geraniol 50%	-	-	+
AOO	8.5	-	-
Ethanol	-652	-	-

Fold of increase are shown as the ratio of proliferation in the test group at each concentration to that in the vehicle control group.
Nd: not done
+ = positive; - = negative; +/- = equivocal.
AOO = acetone-olive oil (4;1, v/v)