

Pathological findings in cumulative irritation induced by SLS and croton oil in hairless mice

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It is known that the pathological features of acute irritant contact dermatitis are specific according to the irritant. However, in chronic irritant contact dermatitis, it is not clear whether specific patterns exist. To investigate whether the specific pathology of acute irritant contact dermatitis is sustained in chronic irritant contact dermatitis, sodium lauryl sulfate (SLS) and croton oil were applied 3× a week for 2 weeks on the dorsal skin of hairless mice using Finn Chambers. The pathologic changes induced by irritants at various concentrations were evaluated using H&E and Luna's staining, as well as immunohistochemistry for 5-bromo-2-deoxyuridine (BrdU), keratin 6 and loricrin. Our results showed that epidermal hyperplasia and inflammatory infiltration were relatively marked in the groups treated with higher concentrations of irritants. These features were more prominent in the 1% croton oil treated group than in the 0.25% SLS treated group. However, lower concentrations of irritants resulted in very similar histological changes, characterized by epidermal hyperplasia with minimal inflammatory infiltration, irrespective of the chemical. Our results suggest that the histological responses to irritants vary with concentration in cumulative irritation, as in acute irritation, but repetitive mild irritation may evoke common histological changes, characterized by epidermal hyperplasia with minimal inflammatory infiltration, irrespective of the chemical used.

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Chronic irritant contact dermatitis (CICD) is a common disorder characterized by persistent dryness, chapping and hyperkeratosis. It is known that CICD is caused by the frequent repetition of subliminal irritation, such that it is also called cumulative irritant contact dermatitis (1). However, the pathogenesis of cumulative irritation by repetitive insult still remains to be elucidated.

In acute irritant contact dermatitis (AICD), histological features show much greater pleomorphism (2) and chemical irritants produce specific pathological changes, which may reflect differences in cytokine production and proliferative response (3, 4). However, it is not clear whether various chemical irritants may produce specific pathological changes in CICD or not. According to a recent report (5) evaluating the chronic toxicity of chemicals, histological changes induced by repetitive application of irritants were extraordinarily diverse. Because of this fact, it was concluded that it was not possible to associate a chemical substance with a specific histological pattern in CICD.

The aim of this study was to determine whether specific pathological findings related to specific chemicals might be found in CICD using hairless mice models. We have investigated the histological changes of acute and cumulative irritation induced by various concentrations of 2 irritants, sodium lauryl sulfate (SLS) and croton oil, which are well known to produce quite different histological changes in acute irritation. Immunohistochemical stains for 5-bromo-2-deoxyuridine (BrdU), keratin 6 and loricrin were also performed to evaluate the proliferating cell population and the alteration of differentiation in the epidermis.

Materials and Methods

Animals

Female hairless mice (SKH-1, Charles River Laboratories, USA) were used. Each group included 5 animals aged 7–8 weeks.

Irritants

SLS (Sigma, USA) and croton oil (Sigma, USA) were used as irritants. Various concentrations that do not cause necrosis of the epidermis were tested as follows. Distilled water and liquid paraffin were applied as controls.

- (i) Concentration of irritants used in single irritation study:
SLS – 0.5%, 0.25%, 0.1%, 0.05%, 0.025% (w/v in distilled water).
croton oil – 4%, 1%, 0.5%, 0.25%, 0.1% (v/v in liquid paraffin).
- (ii) Concentration of irritants used in cumulative irritation study
SLS – 0.25%, 0.05%, 0.025% (w/v in distilled water).
croton oil – 1%, 0.25%, 0.1% (v/v in liquid paraffin).

Application of irritants

(i) Single irritation. 15 μ l of the irritants were put on the filter papers of 8 mm Finn Chambers on Scanpor tape (Epitest Ltd., Finland), which were then applied on the dorsal skin of the animals anesthetized with ketamine and midazolam. Polyurethane film (Tegaderm®, 3 M, USA) was used to fix them and Finn Chambers were then removed 24 h later.

(ii) Cumulative irritation. The procedure was repeated 3 $\times$ a week for 2 weeks.

Evaluations

30 min after removal of the Finn Chambers, skin biopsies were performed with 5 mm punches. Skin biopsies were performed 24 h (day 2) and 72 h (day 4) after removal of the Finn Chambers in the acute irritation study with 0.25% SLS and 1% croton oil. Biopsies were also executed 24 h after removal of the Finn Chambers in the cumulative irritation with 0.05% SLS and 0.25% croton oil.

For evaluation of the proliferating cell population, a solution of 2% BrdU (Sigma, USA) was injected intraperitoneally at a dose of 100 mg/kg 1 h prior to skin biopsy. The specimens were fixed in 10% buffered formalin and processed for haematoxylin and eosin (H&E) stain, Luna's stain for elastic fibres and mast cells (6) and immunohistochemistry. Immunohistochemical staining was performed with the following primary antibodies: anti-BrdU (Dako, Denmark), anti-mouse keratin 6 (Berkley, USA), anti-mouse lor-icrin (Berkley, USA). LSAB® kit (Dako, Denmark) was used for immunohistochemical staining. The sections were developed with 3-

amino-9-ethylcarbazol as a chromogenic substrate.

All sections were observed with a light microscope (BH-2, Olympus, Japan) at 400 $\times$ magnification. Histological parameters evaluated using H&E staining included hyperkeratosis, parakeratosis, spongiosis, exocytosis and dermal infiltration, which were scored as follows: (–) absent, ($\pm$) equivocal, (+) mild, (++) moderate and (+++) severe. The epidermal thickness (excluding horny layer) was measured on 5 to 6 sites for each section. The lengths of stratum corneum were measured. The number of BrdU-positive cells per mm epidermis was calculated.

Statistical analysis

The epidermal thickness and the number of BrdU-labelled cells were analyzed with ANOVA and Turkey's test. Values for *p* less than 0.05 were considered statistically significant.

Results

Visual assessment

In acute irritation, SLS at a concentration of 0.25% or more produced mild flaking, and croton oil at a concentration of 1% or more induced mild to moderate edema with loss of skin creases. In cumulative irritation groups treated with 0.25% SLS and 1% croton oil, the skin showed mild exudation in the 1st week. After repetitive irritation for 2 weeks, all groups showed mild flaking and thickening of the skin. There were no significant changes in body weight of the animals after the experiment.

Histology

(i) *Acute irritation.* In the group treated with 0.5% SLS, the notable histological features were hyperkeratosis, parakeratosis and epidermal hyperplasia. SLS at a concentration higher than 0.5% resulted in epidermal necrosis. Spongiosis and inflammatory infiltrates were not conspicuous (Fig. 1a). A significant increase in epidermal thickness was found in 0.5% and 0.25% SLS-treated groups (Table 1). In the group treated with 4% croton oil, the notable histological features were hyperkeratosis, spongiosis, exocytosis and dense inflammatory cell infiltrates mainly composed of neutrophils (Fig. 1b). Croton oil at a concentration of 8% or more resulted in complete epidermal necrosis. A significant increase in epidermal thickness was also found in the 4% and 1% croton oil-treated groups.

(ii) *Cumulative irritation.* Severe epidermal hyperplasia, spongiosis, neutrophil infiltration and

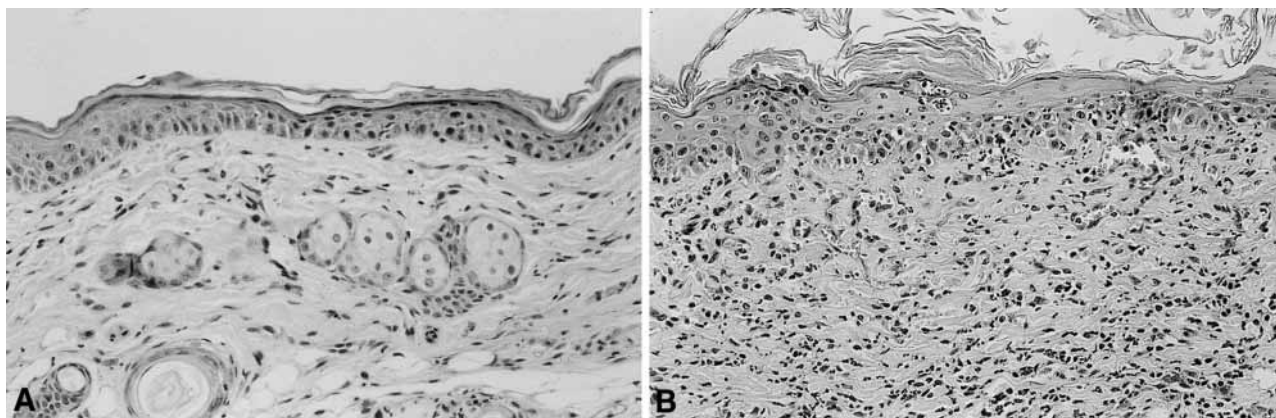


Fig. 1. Histological features of acute irritation (H&E, $\times 200$). A) 0.5% SLS. Hyperkeratosis with parakeratosis and acanthosis of the epidermis are shown. B) 4% croton oil. Hyperkeratosis, spongiosis, exocytosis of the epidermis and dense dermal infiltration mainly composed of neutrophils are observed.

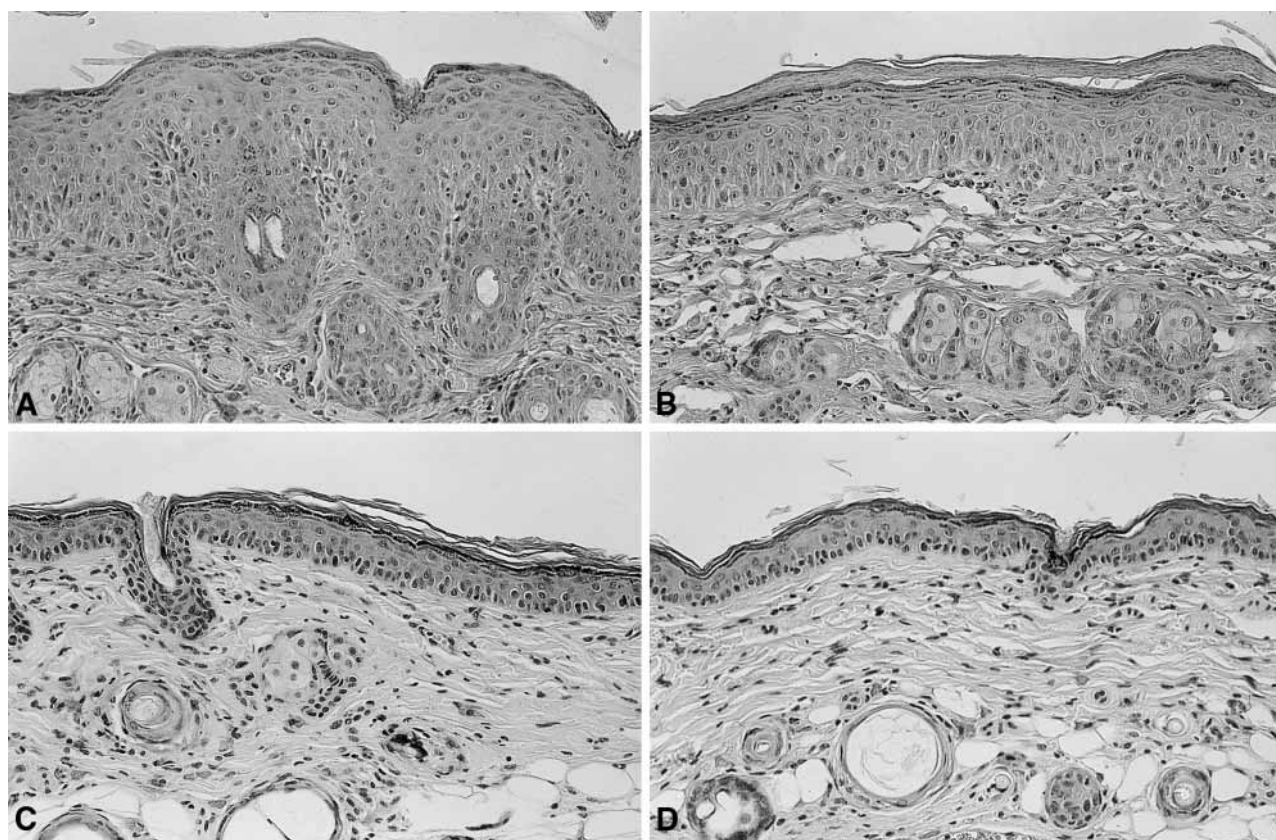


Fig. 2. Histological features of cumulative irritation (H&E, $\times 200$). (A) 1% croton oil. Remarkable features are marked acanthosis with downward growth of the epidermis and dermal inflammatory cell infiltration. (B) 0.25% SLS. Marked hyperkeratosis and acanthosis are observed. (C) 0.1% croton oil. Moderate acanthosis is observed and inflammatory infiltration is sparse. (D) 0.025% SLS. Moderate acanthosis is observed and inflammatory infiltration is sparse.

dilatation of dermal vessels were found in the groups treated with 0.25% SLS and 1% croton oil (Fig. 2a, b, Table 2). However, epidermal hyperplasia and exocytosis were more marked in the 1% croton oil-treated group than in the 0.25% SLS-

treated group. Moreover, a downward growth of epidermis and hypertrophy of the epidermis overlying utriculi was also more evident in the 1% croton oil-treated group.

Epidermal hyperplasia and hyperkeratosis were

also observed in both groups treated with less than 0.25% SLS and 1% croton oil (Fig. 2c, d, Table 2), although the extent was decreased, compared with that at higher concentration. Inflammatory cell infiltration was not conspicuous. Significant differences in histological features were not observed between these 2 irritants at these lower concentrations. Although the control groups treated with liquid paraffin also showed significant epidermal hyperplasia, the extent of epidermal hyperplasia in the groups treated with 1% and 0.25% croton oil was significantly higher than that in the control group. Such a difference was not found in the 0.1% croton oil-treated group. Luna's stain showed increased mast cells and slight increase of elastic fibers in both groups (Fig. 3).

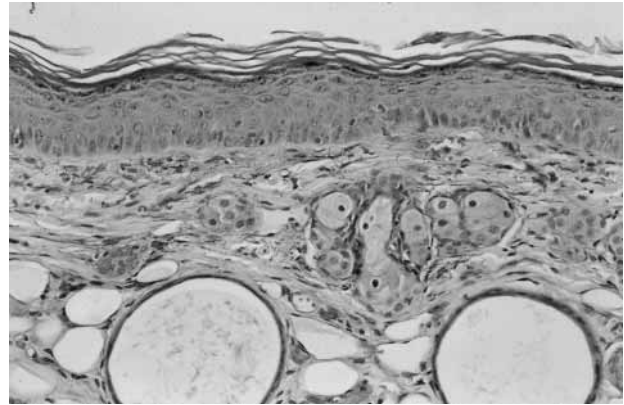


Fig. 3. Luna's stain of cumulative irritation with 0.25% croton oil. Fine elastic fibers are increased and mingled in the upper and middle dermis.

BrdU-labelled cells

In acute irritation, the number of BrdU-labelled cells increased in both groups (Table 3). In cumula-

tive irritation, the number of BrdU-labelled cells was also significantly increased in 1% croton oil- and 0.25% SLS-treated groups by 4.1-fold and 2.8-

Table 1. Histologic features by different doses of irritants in acute irritation

	Hyperkeratosis	Parakeratosis	Spongiosis	Exocytosis	Dermal infiltration	Epidermal thickness
4% croton oil	+++	—	+++	++	+++	43.8±3.47*
1% croton oil	+	—	++	+	+++	36.2±6.66*
0.5% croton oil	±	—	+	—	++	38.3±10.96*
0.25% croton oil	±	—	++	+	+	30.3±5.03
0.1% croton oil	±	—	—	—	±	25.0±3.20
0.5% SLS	+++	++	±	—	+	32.7±4.56*
0.25% SLS	++	+	±	—	±	30.9±4.11*
0.1% SLS	+	—	—	—	—	25.8±2.92
0.05% SLS	±	—	—	—	—	22.9±2.99
0.025% SLS	±	—	—	—	—	22.9±4.10
normal skin	—	—	—	—	—	22.7±3.39
distilled water	—	—	—	—	—	22.9±2.40
liquid paraffin	—	—	—	—	—	24.6±2.48

The histologic parameters, except epidermal thickness, were evaluated semiquantitatively (—=absent, ±=equivocal, +=mild, ++=moderate, +++=severe). The values are those evaluated in the most dominant areas of the histologic sections. The measurements of epidermal thickness represent means and standard deviations.

*Statistically significant difference compared to the normal skin, $p<0.05$.

Table 2. Histologic features by different doses of irritants in cumulative irritation

	Hyperkeratosis	Parakeratosis	Spongiosis	Exocytosis	Dermal infiltration	Epidermal thickness
1% croton oil	++	+	+	+	++	104.7±9.00*
0.25% croton oil	++	—	—	—	+	55.3±3.65*
0.1% croton oil	+	—	—	—	+	38.5±1.17*
0.25% SLS	+++	+	+	—	++	71.6±10.4*
0.05% SLS	++	—	—	—	+	36.0±3.77*
0.025% SLS	+	—	—	—	+	34.6±2.83*
normal skin	±	—	—	—	—	22.7±3.39
distilled water	±	—	—	—	—	25.5±1.27
liquid paraffin	+	—	—	—	±	35.2±1.41*

The histologic parameters, except epidermal thickness, were evaluated semiquantitatively (—absent, ±=equivocal, +=mild, ++=moderate, +++=severe). The values are those evaluated in most dominant areas of the histologic sections. The measurement of epidermal thickness represent means and standard deviations.

*Statistically significant difference compared to the normal skin, $p<0.05$

fold that of acute irritation. However, no significant changes were observed in the groups treated with 0.1% croton oil, 0.05% and 0.025% SLS. In both the acute and cumulative irritation, the number of BrdU-labelled cells more markedly increased on day 2 than on day 1 in SLS-treated groups, whereas croton oil induced a peak increase in the number of BrdU-labelled cells on day 1 (Fig. 4).

Keratin 6 and loricrin expression

Expression of keratin 6 was induced by both irritants at all concentrations. Keratin 6 was expressed in the upper spinous layers. Loricrin stained in the granular layer and there were no differences in the expression patterns of loricrin in acute or cumulative irritation.

Discussion

In acute irritation, we have confirmed that SLS and croton oil produce specific pathologic patterns, as reported in previous studies (3, 4). Occlusion with 4% croton oil for 24 h induced the characteristic histology of severe inflammatory cell infiltration, hyperkeratosis, spongiosis and exocytosis, whereas 0.5% SLS induced less inflammation with hyperkeratosis and parakeratosis. However, these differences were less conspicuous in cumulative irritation. Moreover, lower concentrations of irritants in cumulative irritation resulted in very similar histological changes. Our results suggest that repetitive mild irritation may evoke common histological changes characterized

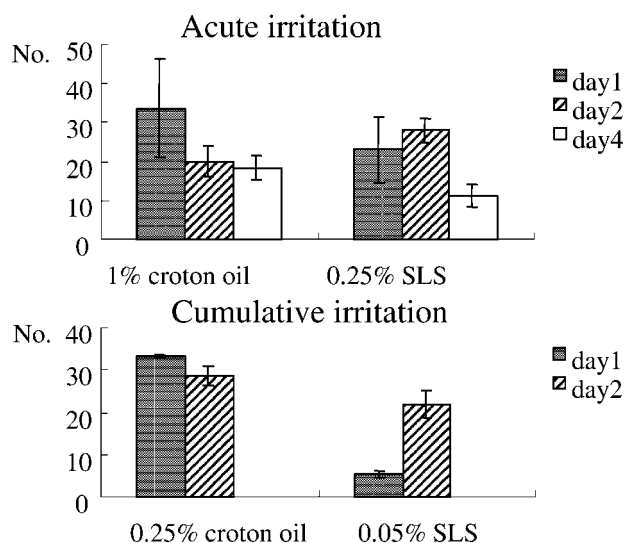


Fig. 4. The numbers of BrdU-labelled cells per mm epidermis were measured at 30 min (day 1), 24 h (day 2) and 72 h (day 4) after removal of the Finn Chambers in acute irritation and cumulative irritation. In both acute and cumulative irritation, the number of BrdU-labelled cells more markedly increased on day 2 than on day 1 in SLS-treated groups, whereas croton oil induced a peak increase in the number of BrdU-labelled cells on day 1.

by epidermal hyperplasia with minimal inflammatory infiltration, irrespective of the chemical being used.

Several studies of the histology of experimentally induced cumulative irritation have been reported (7–10). In these studies, medicaments and cosmetic bases showed different grades of irritancy, however, it was not clearly described whether differences exist according to the chemical nature of irritants. Recently, Kligman (5) tested many kinds of irritants through the method of repeated open application using hairless mice and found that the histological features were heterogeneous depending on the concentration of irritants and their chemical nature. Tavakkol et al. (11) also showed similar results in their study using lower concentrations of SLS. However, our results suggest that repeated exposure to irritants at lower concentration may induce common features irrespective of chemical substances, although the histological responses to irritants showed a tendency to vary with dose even in CICD. This discrepancy may result from the method of application, concentration used and duration of exposure.

Experimental animals such as mice, rats, rabbits (7, 8) and guinea pigs (12) have been used to assess the chronic irritation of chemicals. It was recently reported that experimentation using hairless mice is the most suitable animal model for evaluating the chronic toxicity of chemical irritants (5). In

Table 3. BrdU labelled cells in the groups treated with various concentrations of croton oil and SLS (positive cells/mm epidermis)

Acute irritation		Cumulative irritation	
4% croton oil	50.0±5.0*	1% croton oil	137.6±45.5*
1% croton oil	33.5±12.6*		
0.5% croton oil	19.6±11.5		
0.25% croton oil	29.0±11.6*	0.25% croton oil	33.2±0.4*
0.1% croton oil	26.0±3.0*	0.1% croton oil	12.0±1.8
0.5% SLS	25.0±8.8*		
0.25% SLS	23.0±8.3*	0.25% SLS	64.2±16.0*
0.1% SLS	15.9±3.9		
0.05% SLS	14.1±2.6	0.05% SLS	5.3±0.7
0.025% SLS	11.8±5.6	0.025% SLS	11.8±3.1
normal skin	12.0±4.4	normal skin	12.0±4.4
distilled water	11.3±2.8	distilled water	10.9±4.2
liquid paraffin	14.9±3.5	liquid paraffin	7.2±1.6

The values represent means and standard deviations.

*Statistically significant difference compared to the normal skin, $p < 0.05$.

most studies (5, 7–12), an open application was used because occlusion exaggerates the irritancy of substances. However, open application was reported to produce very diverse histological findings, even in the same section (5). Because of these facts, we used an occlusive method employing Finn Chambers for the application of irritants in hairless mice, rather than an open application. In this study we could observe relatively homogenous and reproducible findings with an occlusive method. Our study also suggests that an occlusive method using Finn Chambers in hairless mice may be one of the most suitable models to study the pathologic changes of irritation.

From the results of the proliferating cell population using BrdU, a striking increase in the number of BrdU-labelled cells was noted in cumulative irritation at relatively high concentrations (0.25% SLS, 1% croton oil), whereas this was not noted in repetitive irritation at lower concentrations. This result suggests that hyperproliferation in CIGD was also grossly related with the concentration of irritant applied.

Croton oil showed peak increase in number of BrdU-labelled cells after removal of Finn Chamber on day 1, whereas it was on day 2 in the case of SLS. This may be due to the character of croton oil, a well-known tumor promoter. A similar result has previously been reported with tumor promoter TPA (12-*O*-tetraacetylphorbol-13-acetate), inducing a more rapid proliferation than another irritant, cantharidin (13).

It is well-known that keratin 6 is a marker of activated keratinocytes. In response to epidermal injury, keratinocytes express a specific pair of keratin proteins, keratin 6/16. It was reported that barrier disruption in murine skin resulted in the induction of keratin 6/16 (14). Our results also show that keratin 6 is induced by both irritants in acute and cumulative irritation. However, loricrin expression, a marker of late-stage differentiation of epidermis, was not altered in this study as it was in a previous report (14). Acute and chronic irritation do not seem to have a great influence on late stage epidermal differentiation.

In conclusion, the repetition of mild irritation may produce common pathologic findings characterized by epidermal hyperplasia with minimal inflammatory cell infiltration, irrespective of the chemical nature of the irritant. It is suggested that mild repetitive irritation may produce CIGD by a common pathologic mechanism different from that of AICD, as was proposed in a previous report (15).

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