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An Alternative Method for Testing Oral Mucosal Irritation Caused by Surfactants

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Summary

Surfactant induced oral mucosal irritation and desquamation were assessed by the two-dimensional *in vitro* test which included neutral red assay, indirect immunostaining for fibronectin and tubulin, western blot for tubulin, and scanning electron microscopic (SEM) evaluation on normal human embryo kerotinocyte monolayers. Mucosal desquamation was also assessed by the three-dimensional *in vitro* test which included light microscopic (LM) and SEM evaluations of a three-dimensional culture of normal human gingival keratinocytes and fibroblasts treated with surfactants. Neutral red assay, and SEM evaluation in the two-dimensional *in vitro* test gave an index of the oral mucosal irritation. And, indirect immunostaining in the two-dimensional *in vitro* test, and LM and SEM evaluations in the three-dimensional *in vitro* test provided an index of desquamation. Benzalkonium chloride treatment resulted in mainly inflammation, while both sodium lauryl sulfate and sodium lauroyl sarcosinate treatments caused mainly desquamation. Tween 20 and Sucrose fatty acid ester did not produce any effects.

It seems that the evaluation of intra- and extra-cellular matrix is simple in the *in vitro* alternative method for oral mucosal desquamation.

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Introduction

The irritation effects of some surfactants on the oral mucosa were reported using hamster cheek pouch mucosa[1)2)], rabbit sublingual mucosa[3)], and dog gingiva[4)].

Human oral mucosa is composed of three types of epithelia: type 1, the alveolar mucosa, the lips and the cheeks; type 2, the gingival epithelium; and type 3, the dorsal part of the tongue. The cytoskeleton consists of three classes of filament-forming proteins: tubulin-containing micro tubules, vitamin-containing intermediate filaments (vimentin filaments, cytokeratin filaments), and actin-containing microfilaments. These cytoskeletons are thought to interact via their associated proteins and involved in many cellular functions including the maintenance of shape, cell mortality, cell adhesion, cell division and organelle localization. The microfilament-regulating proteins which promote or disturb filament formation are integrated into the native cellular structures[5)]. Certain substances affect the growth of cultured cells and produce cytoskeletal alterations. These have been used as parameters for toxicity assessment[6)]. We considered that subtle irrita-

tion to the oral mucosa may be estimated by evaluating the changes of cytoskeletal organization and extracellular matrix. On the other hand, a three-dimensional *in vitro* model for estimating oral irritation using submucosal connective tissue in rabbits was reported[7]. As an alternative model for assessing oral mucosal irritation, we performed neutral red assay as a test for cell injury, immunostaining for tubulin and fibronectin, western blot for tubulin, and SEM evaluation as tests for cell desquamation, using human embryo keratinocyte monolayers. And, the three-dimensional *in vitro* test by LM and SEM evaluations was performed on a three-dimensional culture model of human gingival keratinocytes and fibroblasts.

Materials and Methods

Cell cultures

For the two-dimensional *in vitro* test, human epidermal keratinocytes were derived from explant cultures of a human embryo. Keratinoytes used in these experiments were at passages four to five. They were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% foetal calf serum, in a humidified atmosphere of 5% CO_2 in air at 37°C. Culture media were obtained from Nissui Seiyaku Co., Ltd., Tokyo, Japan.

For the three-dimensional *in vitro* test, human gingival keratinocytes and fibroblasts were isolated from a piece of gingival tissue obtained from a healthy volunteer, who was operated for the extraction of wisdom tooth in Sunstar Foundation for Dental Health Promotion. The keratinocytes and fibroblasts used in these experiments were at passages four and seven, respectively. Keratinocytes and fibroblasts were grown in Serum-free keratinocyte medium (SFM) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, in a humidified atmosphere of 5% CO_2 in air at 37°C, respectively. SFM was obtained from Gibco Laboratories, USA. DMEM was purchased from Nissui Seiyaku Co., Ltd., Tokyo, Japan.

Chemicals

The anionic surfactants, sodium lauryl sulfate (SLS, 94.5% purity) and sodium lauroyl sarcosinate (LS, 90.0% purity) were used. The cationic surfactant, benzalkonium chloride (BC, 99.5% purity) was used. The nonionic surfactants, polyoxyethylene, (20) sorbitan monolaurate (Tween 20, 99.0% purity) and sucrose fatty acid ester (SE, 95.3% purity) were used.

Anti-tubulin monoclonal antibody, fluorescein-conjugated anti-mouse Ig, biotinylated anti-mouse Ig, and streptavidin/biotinylated alkaline phosphatase were obtained from Amersham International Plc, UK. Anti-fibronectin monoclonal antibody was purchased from Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan. BCIP (5-Bromo-4-chloro-3-indolyphosphate p-Toluidine Salt) and NBT (Nitroblue Tetrazolium Chloride) were obtained from Gibco BRL, USA. Type I collagen gel was from Koken, Tokyo, Japan.

Two-dimensional in vitro test:
Cell injury tests (Neutral red assay)

The human epidermal keratinocytes were inoculated into the wells (4×10^4 cells/ml, 0.1 ml per well) of a 96-well tissue culture plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan), and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C for 3 days. The medium was then changed from EMEM containing 10% foetal calf serum to serum-free EMEM containing insulin (1 mg/litre), transferrin (10 mg/litre) and epidermal growth factor (EGF) (0.01 mg/litre), and the cells were then treated with a range of surfactants diluted in serum-free medium for 2 days. The detergents were used in wells of six without sterilization. After treatment, the cultured media were removed, the cells were washed with $CaCl_2$- and $MgCl_2$-free phosphate buffered saline (PBS(−)). 0.1 ml EMEM supplemented with neutral red (50 μg/ml) was added to each well. The cells were

then incubated at 37°C for 3 hr. Neutral red media were removed, the cells were washed with PBS(−) and fixed with 1% formaldehyde, and then 0.1 ml of 1% acetic acid-50% isopropanol was added to each well for 20 min. Absorbance at 540 nm was measured with a microplate reader (NJ 2000, Inter Med), and NR50 (compound concentration causing 50% cell death) was calculated.

Cell desquamation test

3×10^4 cells were inoculated on inserted cover slips (10×10 mm) placed in wells of a 24-well tissue culture plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and cultured, in a himidified atmosphere of 5% CO_2 in air at 37°C for 3 days. The media were then removed, and the cells were treated for 10 min with 0.05% and 0.10% surfactants diluted in EMEM.

For indirect immunostaining, cultured media were removed and cells were washed with PBS(−) and fixed by exposure to absolute methanol at −20°C for 5 min. The cells were washed with PBS(−) and incubated with primary monoclonal antibodies, at 37°C for 1 hr. The primary monoclonal antibodies used were anti-tubulin (1:500), and anti-fibronectin (1:5) diluted in Tris buffered saline (TBS). Cells were washed three times in PBS(−) and incubated with a fluorescein-conjugated anti-mouse Ig at 37°C for 30 min. The primary antibody was visualized by fluorescence microscopy.

For western blot, after the cultured medium was removed, the cells were washed with PBS(−), lysed in RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% NP40, 1% Sodium deoxycholate, and 0.05% SDS and analyzed by a one-dimensional sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis (SDS-PAGE) on 10% gels with standard polypeptide molecular weight markers[8].

The proteins were electroblotted onto polyvinyl difluoride membrane at 200 mA for 1 hr. The transferred membrane was blocked with 7% dry milk at room temperature (r.t.) overnight. After the membrane was incubated with anti-tubulin (1:1000 in TBS) at r.t. for 1 hr, it was incubated with biotinylated anti-mouse Ig (1:500 in TBS) and streptavidin/biotinylated alkaline phosphatase (1:3000 in TBS) at r.t. for 1 hr and 30 min, respectively. The membrane was then visualized with BCIP (5-Bromo-4-chloro-3-indolyphosphate p-Toluidine Salt) and NBT (Nitroblue Tetrazolium Chloride). Then it was washed with distilled water and dried. The results were analyzed densitometrically with a dual-wavelength flying-spot scanner (CS-9000, Shimadzu) and expressed as the % area of the untreated control.

For scanning electron microscopic (SEM) observation, cells were fixed in 2.5% glutaraldehyde, dehydrate with ethanol and butanol, and coated with platinum after drying.

Three-dimensional in vitro test:
Cell desquamation test

Human gingival fibroblasts were cultured in 0.2% type I collagen gel in culture medium (10^5 cells/ml, 1 ml per 35 mm dish), and geled in a humidified atmosphere of 5% CO_2 in air 37°C for 7 days. After aggregation to about 20% of the initial dimension, human gingival keratinocytes (2×10^4 cells/cm^2) were inoculated on the human gingival fibroblast gel, and cultured for 2 weeks in a mixed media containing equal volume of DMEM and SFM with 10% foetal calf serum. The medium was then removed, and the cultures were treated with 0.05% surfactants diluted in SFM for 10 min.

For light microscopic (LM) observation, the three-dimensional cell cultures were fixed in 10% neutral buffered formalin, sectioned at 5 μm thickness and stained with hematoxylin and eosin.

For scanning electron microscopic (SEM) observation, the three-dimensional cell cultures were fixed in 10% neutral buffered formalin, dehydrated with ethanol and butanol, and coated with platinum after drying.

Results

Two-dimensional in vitro test

Cell injury test

The results of the cell injury test are shown in table 1. The rank of the degree of cell injury caused by the surfactants was BC > SLS, LS > SE, Tween 20.

Cell desquamation test

Indirect immunostaining was used to assess fibronectin and tubulin structures (see Plate 1-1 and 1-2). SLS at 0.05% induced a total disappearance of the fibronectin meshwork, and decrease and reinforcement of tubulin filament; and at 0.1% caused the disappearance of both the fibronectin meshwork and tubulin filament. LS at 0.05% induced breaking of the fibronectin meshwork, and reinforcement of tubulin filament; and at 0.1% caused the total disappearance of the fibronectin meshwork and tubulin filament. Cells treated with 0.1% of BC still maintained an almost intact fibronectin meshwork and tubulin filament, but their shape was changed from fusiform to sphere. Tween 20 and SE even at 0.1% did not change the shape of fibronectin meshwork and tubulin filament.

The results of the Western blot are shown plate 2 and table 1. The amount of tubulin was quantitated densitometrically and the result is expressed as the percent optical density of the untreated control. The rank of the decrease in quantity of tubulin filament caused by the surfactants was SLS (4%) > BC (45%) > SE (73%), LS (82%), Tween 20 (82%).

SEM observations are shown in plate 3-1 and 3.2. The control was an intact monolayer. SLS treatment resulted in desquamation of all the cells. Although the monolayer was still present after LS treatment, disappearance of extracellular substance and distinct individual cells were observed. Although the monolayer was maintained after BC treatment, the cell wall and plasma membrane were resolved, and the cytoskeleton was exposed. Treatment

Table 1. Effects of surfactants on cultivated normal human keratinocytes and hamster cheek pouch mucosa

Methods	Two-dimensional *in vitro* test*							Three-dimensional *in vitro* test‡		*In vivo* test‡‡			
	NR assay§	Indirect immunostaining				Western blot§§							
	NR50	Fibronectin		Tubulin		Tubulin	SEM¶	LM¶¶	SEM¶	LM¶¶		SEM¶	
Samples	(μg/ml)	0.05%	0.10%	0.05%	0.10%	0.05%	0.05%	0.05%	0.05%	1.0%	10.0%	1.0%	10.0%
SLS	21.90± 1.56	+++	+++	+	+++	4	+++	+++	+++	+	++	+	++
LS	23.81± 1.65	±	+++	±	+++	82	±	++	++	–	±	++	+++
BC	3.24± 0.51	±	±	±	±	45	++	–	–	++	+++	±	+
Tween 20	398.87±26.48	±	±	–	–	82	–	–	–	–	±	–	±
SE	128.33± 7.39	–	–	–	–	73	–	±	±	–	±	–	±

SLS: Sodium lauryl sulfate LS: Sodium lauroyl sarcosinate BC: Benzalkonium chloride Tween 20: Polyoxyethylene (20) sorbitan monolaurate SE: Sucrose fatty acid ester

– : negative ±: slightly positive +: positive ++: moderately positive +++: strongly positive

* Two-dimensional *in vitro* test consisted of the neutral red (NR) assay as a cell injury test, and immunostaining, western blot, and scanning electron microscopic (SEM) observation as cell desquamation tests, using normal human embryo keratinocytes.

‡ Three-dimensional *in vitro* test evaluated cell desquamation by light microscopic (LM) and scanning electron microscopic (SEM) observations.

‡‡ The *in vivo* test was the oral mucosal irritation test using hamster cheek pouch reported by Yoshikawa (1978).

§ NR50 means compound concentration causing 50% cell death.

§§ Western blot indicated the percent optical density of untreated control analyzed densitometrically.

¶ SEM means scanning electron microscopic observation.

¶¶ LM means light microscopic observation.

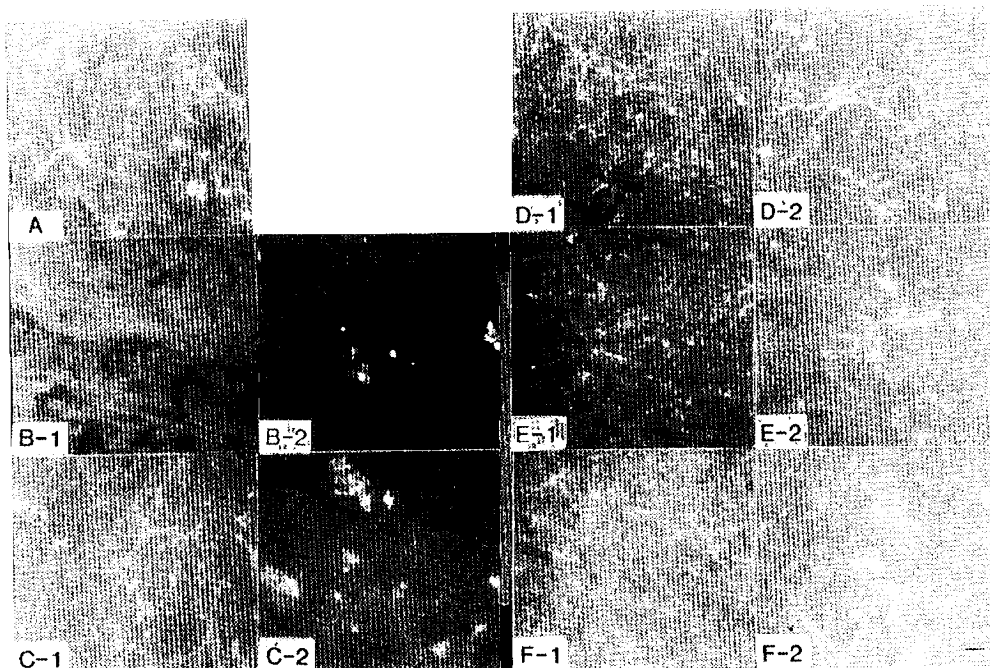

Plate 1-1 Immunofluorescence staining for fibronectin in normal human embryo keratinocytes exposed to 0.05 and 0.10% surfactants for 10 min. A=control; B-1=0.05% Sodium lauryl sulfate (SLS); B-2=0.10% SLS; C-1=0.05% Sodium lauroyl sarcosinate (LS); C-2=0.10% LS; D-1=0.05% Benzalkonium chloride (BC); D-2=0.10% BC; E-1=0.05% Polyoxyethylene (20) sorbitan monolaurate (Tween 20); E-2=0.10% Tween 20; F-1=0.05% Sucrose fatty acid ester (SE); F-2=0.10% SE; Bar =20 μm.

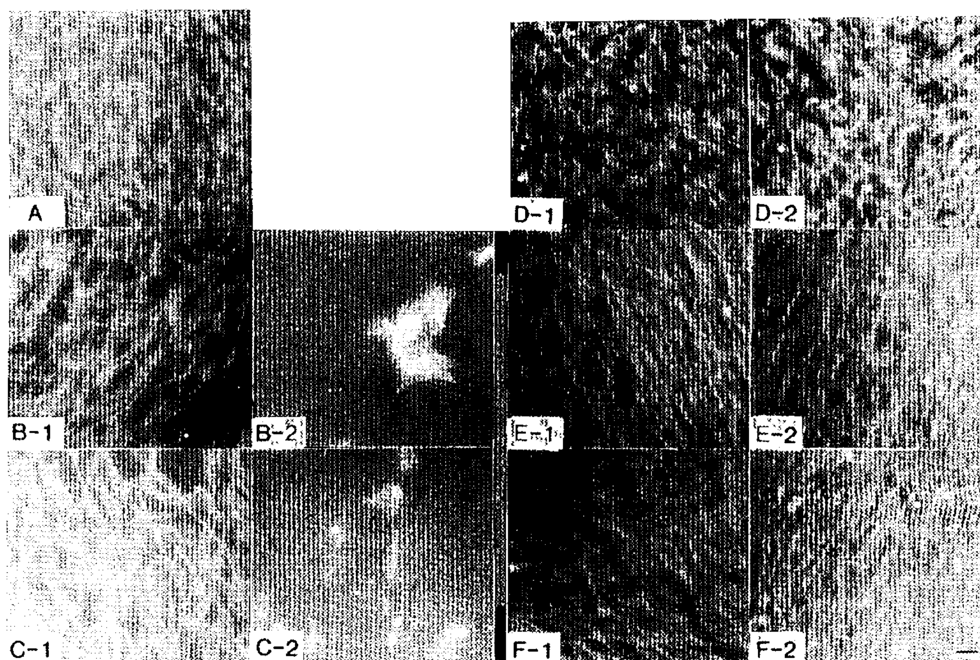

Plate 1-2 Immunofluorescence staining for tubulin in normal human embryo keratinocytes exposed to 0.05 and 0.10% surfactants for 10 min. A=control; B-1=0.05% Sodium lauryl sulfate (SLS); B-2=0.10% SLS; C-1=0.05% Sodium lauroyl sarcosinate (LS); C-2=0.10% LS; D-1=0.05% Benzalkonium chloride (BC); D-2=0.10% BC; E-1: 0.05% Polyoxyethylene (20) sorbitan monolaurate (Tween 20); E-2=0.10% Tween 20; F-1=0.05% Sucrose fatty acid ester (SE); F-2=0.10% SE; Bar=20 μm.

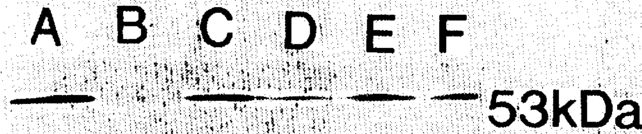

Plate 2 Western blot demonstrating tubulin in normal human embryo keratinocyte proteins exposed to 0.05% surfactants for 10 min. Proteins were electrophoresed on 10% SDS-polyacrylamide gel and blotted onto polyvinyl difluoride. A=control; B=Sodium lauryl sulfate; C=Sodium lauroyl sarcosinate; D=Benzalkonium chloride; E=Polyoxyethylene (20) sorbitan monolaurate; F=Sucrose fatty acid ester. Each lane indicates the proteins obtained per cover slip.

by Tween 20 and SE caused the development of microfibril and expanded cells.

Three-dimensional in vitro test

Cell desquamation test

LM and SEM observations are shown in plate 4, plate 5-1 and plate 5-2. The control showed one to three layers of human gingival keratinocyte on the type I collagen matrix cultivated human gingival fibroblasts. SLS treatment resulted in the stripping of the keratinocyte layers, leaving only the type I collagen matrix. LS and SE treatments resulted in cell desquamation in places. BC and Tween 20 treatments did not affect the keratinocyte layers.

Discussion

Table 1 summarizes the results of the two- and three-dimensional *in vitro* tests of this study, and the results of the *in vivo* test using hamster cheek pouch mucosa reported by Yoshikawa[1]. By histological study *in vivo*, Yoshikawa[1] reported that surfactants could exert two modes of action on the oral mucosa, irritation and desquamation.

The LM evaluation in the *in vivo* test using hamster mucosa was used as an index of inflammation, the rank of the degree of inflammation was BC > SLS > LS, SE, Tween 20, which agrees with the results of neutral red assay and SEM evaluation in the two-dimensional *in vitro* test in this study.

The SEM evaluation in the *in vivo* test was used as an index of desquamation, the rank of the degree of desquamation was LS, SLS > BC > Tween 20, SE, which coincides with the results of the indirect immunostaining for fibronectin and tubulin in this study. The quantities of tubulin detected densitometrically in the Western blot disagree with those assessed by the naked eye in the indirect immunostaining. These results illustrate that the indirect immunostaining demonstrates not only the quantity of tubulin but the strength of fluorescence and the change of cytoskeleton. It seems that the cell damages caused by surfactants in the *in vitro* models are mainly cell detachment, polymerization of tubulin filaments and decrease of fibronectin meshwork. In the immunostaining study, the cells used were human embryo keratinocytes. Human gingival keratinocytes organize tubulin, actin, and keratin[9], while they have little organized fibronectin meshwork (data not shown).

The LM and SEM evaluations using the three-dimensional gingival model agree with the result of SEM evaluation using hamster cheek pouch mucosa. However the results of BC treatment observed by SEM were different in the two- and three-dimensional *in vitro* tests. There was little cell desquamation in the three-dimensional *in vitro* test, in spite of the demonstration of exposure of cytoskeleton in

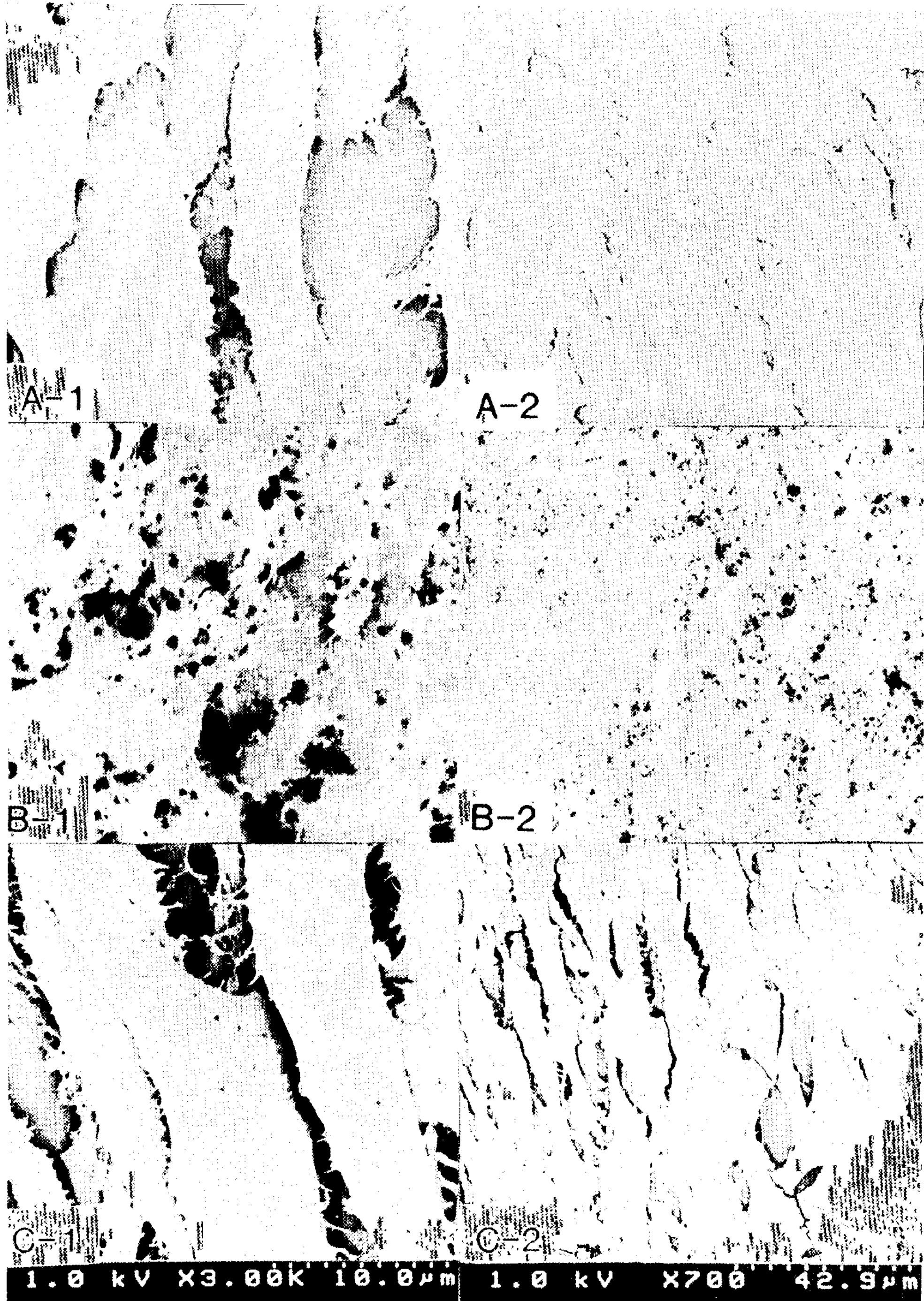

Plate 3-1 Scanning electron microscopy (SEM) of normal human embryo keratinocytes exposed to 0.05% surfactants for 10 min. A-1=control, ×3,000; A-2=control, ×700; B-1=Sodium lauryl sulfate (SLS), ×3,000; B-2=SLS, ×700; C-1=Sodium lauroyl sarcosinate (LS), ×3,000; C-2=LS, ×700.

Plate 3-2 Scanning electron microscopy (SEM) of normal human embryo keratinocytes exposed to 0.05% surfactants for 10 min. D-1=Benzalkonium chroride (BC), ×3,000; D-2=BC, ×700; E-1=Polyoxyethylene (20) sorbitan monolaurate (Tween 20), ×3,000; E-2=Tween 20, ×700; F-1=Sucrose fatty acid ester (SE), ×3,000; F-2=SE, ×700.

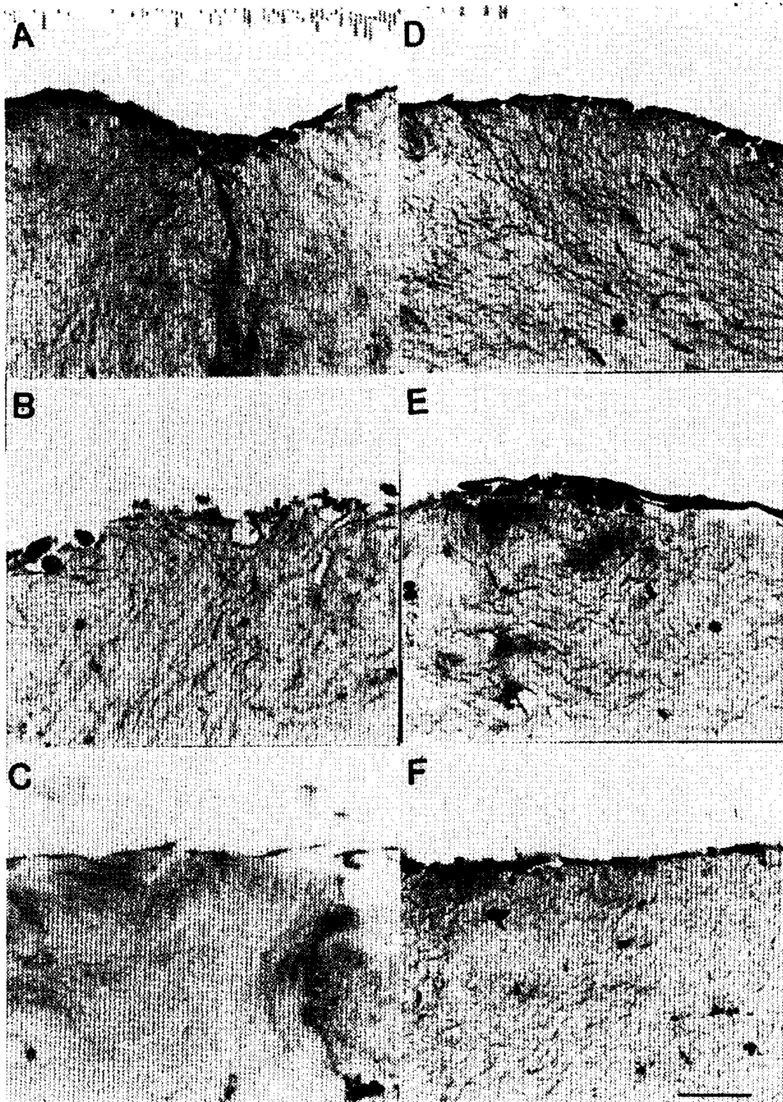

Plate 4 Light microscopy (LM) of a three-dimensional gingival model exposed to 0.05% surfactants for 10 min. A=control; B=Sodium lauryl sulfate; C=Sodium lauroyl sarcosinate; D=Benzalkonium chloride; E=Polyoxyethylene (20) sorbitan monolaurate; F=Sucrose fatty acid ester; Bar=50 μm.

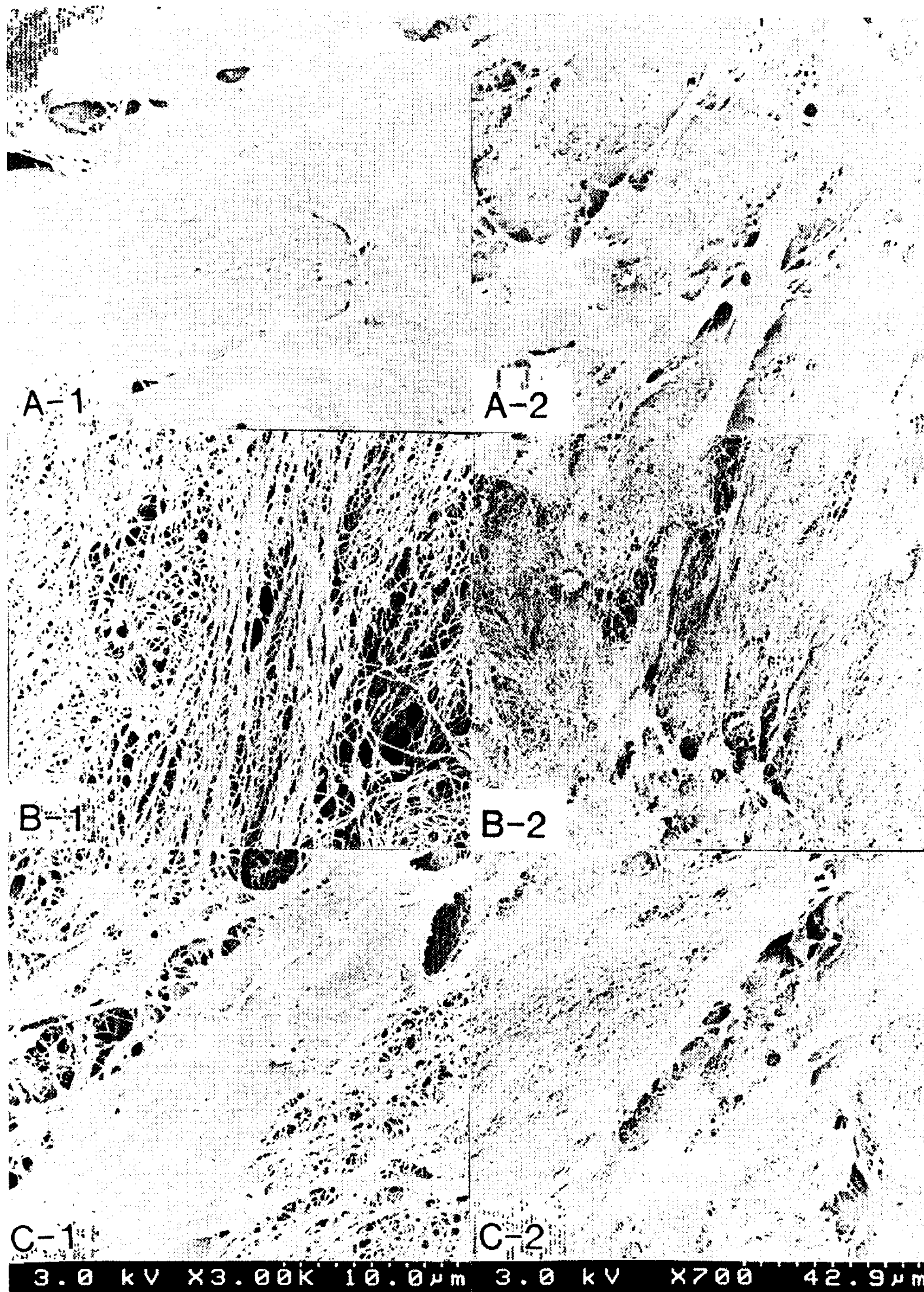

Plate 5-1 Scanning electron microscopy (SEM) of a three-dimensional gingival model exposed to 0.05% surfactants for 10 min. A-1=control, ×3,000; A-2=control, ×700; B-1=Sodium lauryl sulfate (SLS), ×3,000; B-2=SLS, ×700; C-1=Sodium lauroyl sarcosinate (LS), ×3,000; C-2=LS, ×700.

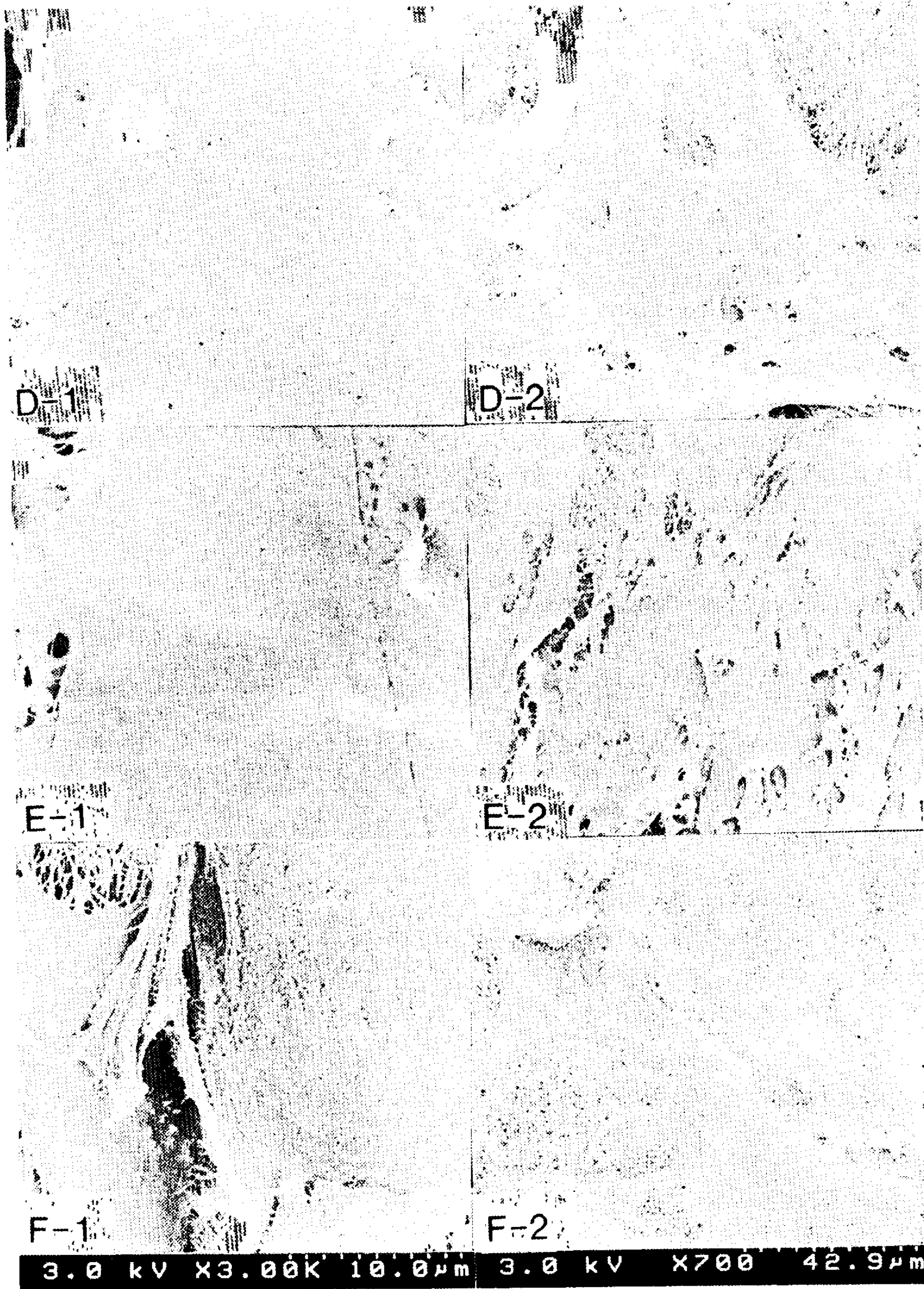

Plate 5-2 Scanning electron microscopy (SEM) of a three-dimensional gingival model exposed to 0.05% surfactants for 10 min. D-1=Benzalkonium chloride (BC), ×3,000; D-2=BC, ×700; E-1=Polyoxyethylene (20) sorbitan monolaurate (Tween 20), ×3,000; E-2=Tween 20, ×700; F-1=Sucrose fatty acid ester (SE), ×3,000; F-2=SE, ×700.

the two-dimensional *in vitro* test. The discrepany may be due to the following: (1) BC (cationic surfactant) is adsorbed by type I collagen matrix having a negative charge, (2) the cells in the three-dimensional culture behave differently to those in the two-dimensional culture, (3) the origins of cells for the two tests were different. The *in vitro* test using cultured cells could not express phenomena (i.e., longestion, hemorrhage, microabscess, infiltrate) of mucosal irritation shown in the hamster cheek pouch[10]. Ouhayoun et al.[11] reported that the buccal gingiva in human is different from the alveolar mucosa morphologically. Therefore the oral mucosal irritation and desquamation tests should be assessed in epithelial cell from the oral mucosa.

It seems that the evaluation of intra- and extra-cellular matrix is simple in the *in vitro* alternative method for oral mucosal desquamation. The results of the cell injury test coincide with those of the *in vivo* oral mucosal inflammation test for a range of surfactants with different characters. However, it is doubtful that the cell injury test, which distinguishes dead and living cells is relevant in indicating slight inflammation and desquamation in skin and oral mucosa.

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