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Surfactant-Induced Alteration of Arachidonic Acid Metabolism of Mammalian Cells in Culture (42503)

VINCENT A. DE LEO,* LEONARD C. HARBER,* BETTY M. KONG,† AND SALVATORE J. DE SALVA†

Department of Dermatology, Columbia University, 630 West 168th Street, New York, New York 10032, and †Colgate-Palmolive, Piscataway, New Jersey 08854

Abstract. Primary irritancy in human and animal skin is characterized by an inflammatory reaction mediated, in part, by membrane-derived arachidonate metabolites. One of the mechanisms of this reaction was investigated in cultured mammalian cells using three surfactants: linear alkyl benzene sulfonate (LAS), alkyl ethoxylate sulfate (AEOS), and TWEEN 20. These compounds listed in order *in vivo* irritancy are LAS > AEOS > TWEEN 20. Each of these compounds was studied in C3H-10T1/2 cells and human keratinocytes which had been prelabeled with 3H-labeled arachidonic acid (AA). After labeling, media were removed, cells were washed, and fresh media with or without surfactant were added. Cells were then incubated for 2 hr, media were removed and centrifuged, and an aliquot was assayed by liquid scintillation for release of label. In C3H-10T1/2 cells LAS and AEOS in 5–50 μM concentration stimulated 2 to 10 times the release of [^{3}H]AA as compared to controls. In contrast, concentrations of 50–100 μM of TWEEN were required to release [^{3}H]AA. With keratinocytes the same rank order of surfactant concentrations necessary for release was obtained as found with C3H-10T1/2 cells. High-performance liquid chromatography of media extracts of both cell systems revealed surfactant stimulation of the production of cyclooxygenase AA metabolites. These results confirm the induction of release by primary irritants of fatty acid groups from membrane phospholipids. Subsequent metabolism of these fatty acid groups are an integral part of the primary irritant response. Data presented with three known irritants in this *in vitro* model show a direct correlation with *in vivo* studies.

Dermatitis due to the direct toxic effect of chemical agents on human skin is common. Among workers in industry, skin disease accounts for approximately one-half of all job-related morbidity, and it is in this group that the "primary irritant reaction" is the most frequently encountered pattern of clinical pathology (1–3). Such pathology is not limited to industry. Many reactions classified by clinicians under a myriad of terms, including "housewives eczema" and "diaper dermatitis," are primary irritant reactions (4). Despite the prevalence of such reactions little is known about the mechanisms of their production.

The primary irritant response in human and animal skin is evidenced initially by the classic signs of inflammation, including erythema and edema. The ability of metabolites of arachidonic acid (AA) to mediate this type of localized vascular response in human skin suggests that the cell membrane may be a site of interaction for many cutaneous irritants. In fact, increased levels of the arachidonic acid derived prostaglandins PGE_2 and $PGF_{2\alpha}$ have been recovered from the skin of experimental animals and humans treated topically with the irritants carrageenan and benzalkonium chloride (5–7).

A variety of chemical substances, including structurally simple acids and bases as well as more complex agents like phorbol esters, are capable of illiciting such responses in human and animal skin. Although no statistics exist detailing the frequency of a given group of agents in the etiology of irritant dermatitis it is likely that some soaps and detergents in marketed consumer products account for a large percentage of irritant reactions in the general population.

Cells in culture have proven to be excellent models for exploring the mechanisms by which membrane toxins, including phorbol esters, induce alterations in cellular metabolism (8). We have used two such systems to examine the effect of known irritants on cellular arachidonic acid metabolism. The irri-

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tants chosen for study were three surfactants which have a defined *in vivo* irritancy in animal skin (9). In addition these agents are components of a wide variety of presently marketed consumer products.

Materials and Methods. *Cell culture systems: C3H-10T1/2.* Cells of this mouse embryo fibroblast line were maintained in growth medium—Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (1 mg/ml), and fungizone (0.25 μg/ml). Cells were grown in dishes at 36°C in water-saturated atmosphere of 5% CO_2 in room air. Cells were seeded at approximately 2×10^4 cells/cm^2. Cells were utilized between passages 9 and 12.

Human epidermal keratinocytes. Human epidermal keratinocytes cultures were prepared according to the methods of Eisinger *et al.* (10). Briefly, breast skin from mastectomy specimens was cut into small discs and incubated overnight in a 0.25% trypsin solution at 4°C. The epidermis of each disc was peeled off of the dermis and pooled in a trypsin/EDTA solution to prepare a single cell suspension. The suspension was counted and appropriately diluted in growth medium—minimal essential medium with Earle's salts supplemented with 10% fetal calf serum, and penicillin (100 units/ml), streptomycin (1 mg/ml), fungizone (0.25 μg/ml), nonessential amino acids (0.01 m*M*), and L-glutamine (2 m*M*). Cells were seeded into dishes at 1.4×10^5 cells/cm^2. Cells were maintained at 36°C in moisturized 5% CO_2 with room air. Cells were used for these studies in primary or first passages.

Materials. Linear alkyl benzene sulfonate (LAS), alkyl ethoxylate sulfate-3EO (AEOS), and TWEEN 20 were obtained from Colgate–Palmolive (Piscataway, NJ). Media, fetal calf serum, and media antibiotics and other supplements were obtained from GIBCO. ^{3}H-labeled arachidonic acid (93 Ci/mmol; 1 mCi/1 ml) was obtained from Amersham. Standards for prostaglandins were a generous gift of the Upjohn Corp. (Kalamazoo, MI).

Experimental procedure. Cells were seeded into dishes and allowed to attach and grow under the conditions outlined above. After 24 hr (C3H-10T1/2) or 9 to 12 days (human keratinocytes) the cells were prelabeled with ^{3}H-labeled arachidonic acid in growth medium (1 μCi/ml) for a 24-hr period under routine incubation conditions. After the labeling period, the media were removed and the cells were washed three times with growth medium (without fetal calf serum). Growth medium (without fetal calf serum) with or without (control) surfactant was then added to the dishes. The cells were incubated for selected periods of time. The media were removed and centrifuged at 12,000 rpm to remove detached cells, and an aliquot was assayed for radioactivity by liquid scintillation spectroscopy. The data calculated in terms of cpm/dish represent the total release of label and consist of all labeled arachidonate metabolites as well as true arachidonic acid. All data points are the means of triplicate dishes (SEM $\leq$ 10%). Data were examined for statistical significance utilizing the Student *t* test.

Determination of arachidonic acid metabolite production. In order to determine surfactant-induced production of specific arachidonic acid metabolites, media from a number of surfactant-treated or control dishes were pooled. Metabolites were extracted according to the techniques (11). The media were acidified and vortexed with ethyl acetate. The ethyl acetate layers from three such extractions were pooled and dried under nitrogen. The dried extracts were redissolved in ethanol and examined by high-performance liquid chromatography (HPLC). The HPLC system has been previously described (12) and consisted of a reverse-phase, gradient elution of acetonitrile and water (0.1% acetic acid). Fractions of eluate were collected at 1-min intervals. Radioactivity in each fraction was determined by liquid scintillation and identity of each of these peaks was confirmed by collection of eluate and coelution of radioactivity in a thin-layer chromatography system with arachidonic acid metabolite standards (11). The standards included arachidonic acid, thromboxane B_2, 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, and PGD_2.

Results. The data presented in Fig. 1 are representative of a typical dose–response experiment in which prelabeled C3H-10T1/2 cells were treated with each of the three surfactants and incubated for a 2-hr period. The release of label from surfactant-treated cells increased with increasing concentration of surfactant for the three agents tested. The magnitude of the response was greater in cells

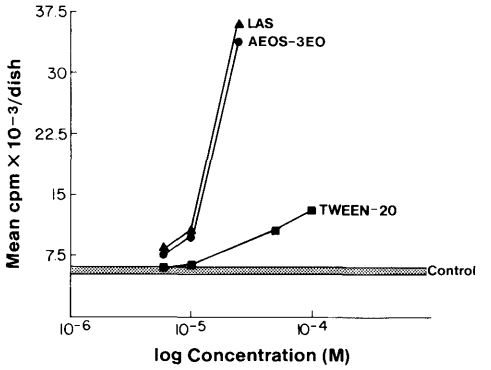

FIG. 1. Release of label. C3H 10T1/2 cells were grown in dishes and prelabeled with [^{3}H]arachidonic acid. The cells were then treated with varying concentrations of three surfactants for a 2-hr period. Media were removed from treated and control cells and an aliquot was assayed for radioactivity. Data presented represent means of triplicate dishes.

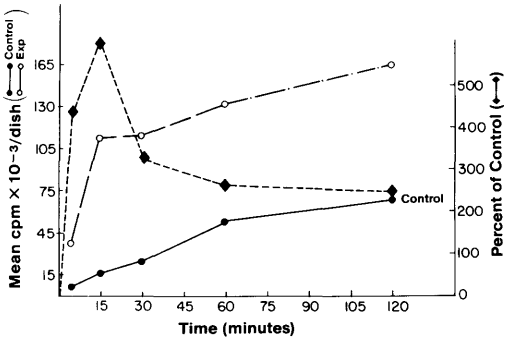

FIG. 2. C3H 10T1/2 cells were grown in dishes and prelabeled with [^{3}H]arachidonic acid. Cells were treated with AEOS, 3×10^{-5} M in medium or medium alone (control). Media from treated and control dishes were removed at selected times after treatment. (○) Surfactant; (●) control; (◆) "percentage of control" = cpm/dish surfactant treated cells cpm/dish control cells. All data points represent means of triplicate dishes.

treated with LAS and AEOS than in cells treated with TWEEN 20. The rank order of response in Fig. 1 in terms of release of label correlates positively with the rank order of irritancy of these three agents in animal skin: LAS > AEOS > TWEEN 20 (9, and B. Kong, unpublished observation). The differences in response (release of label) between LAS and TWEEN 20 and between AEOS and TWEEN 20 were both statistically significant at surfactant concentration of $5 \times 10^{-6}M$ ($P \leq 0.05$). In addition, statistical comparison of the responses to the $1 \times 10^{-5}M$ concentrations of TWEEN 20 yielded significance at $P \leq 0.01$.

To determine the time course of the release of label induced by surfactants, prelabeled C3H-10T1/2 cells were treated with a single concentration of one of the surfactants (AEOS, 3×10^{-5} M). Media were removed for assay from both surfactant-treated and control cells at selected times after treatment. The data from such an experiment are presented in Fig. 2. It is apparent that the maximal surfactant-induced release of arachidonate metabolites from these cells in culture occurred within the 15-min period following surfactant treatment.

When prelabeled human keratinocytes were treated with the surfactants, release of label again correlated with the concentration of each surfactant (Fig. 3). The response to a given concentration of the three agents resulted in the same rank order as that found in the C3H-10T1/2 system: LAS > AEOS > TWEEN 20. The differences between the responses to LAS and TWEEN 20 were statistically significant ($P \leq 0.05$) at the highest concentration tested.

Media from surfactant treated and control cultures of both cell systems prelabeled with ^{3}H-labeled arachidonic acid were removed after a 2-hr incubation and the media from three dishes of each group were pooled. Media extracts were examined by HPLC to identify specific arachidonate metabolites synthesized.

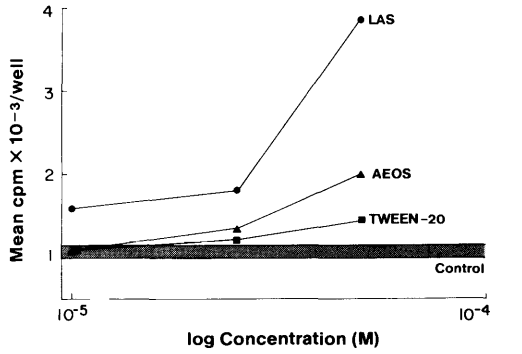

FIG. 3. Release of label. Human keratinocytes were grown in dishes and prelabeled with [^{3}H]arachidonic acid. Cells were treated with surfactants in medium or with medium alone. After a 2-hr incubation, media were removed and assayed for release of radioactive label. Data represent means of triplicate dishes.

TABLE I. SURFACTANT-INDUCED PRODUCTION OF ARACHIDONATE METABOLITES BY C3H 10T1/2 CELLS, AEOS (1.5×10^{-5} *M*)

Metabolite	cpm/dish		Ratio, surfactant/ control
	Control	Surfactant	
$6KPGF_{1\alpha}$	200	1110	5.35
$PGF_{2\alpha}$	240	635	1.90
PGE_2	230	455	2.80
Arachidonic acid	1720	6185	3.60
Total	2390	8385	3.51

Note. C_3H 10T1/2 cells were prelabeled with ^{3}H-labeled arachidonic acid and treated with AEOS (1.5×10^{-5} *M*) in growth medium without fetal calf serum or with medium alone. After 2-hr, media from surfactant-treated and control cells were extracted and the extracts were examined by HPLC for identification of ^{3}H-labeled arachidonate metabolites.

The data from these experiments are presented in Tables I and II. (The concentration of surfactant used in each system is detailed in the tables and was chosen so as to result in a release of label of approximately two to three times control levels.)

Control cells of the C3H-10T1/2 line were found to produce radioactively labeled metabolites which eluted with standards for 6-keto-$PGF_{1\alpha}$ (prostacyclin derivative), PGE_2, and $PGF_{2\alpha}$. No radioactivity above background was found to elute with standards for thromboxane and PGD_2. Surfactant treatment resulted in stimulation of production of the same three metabolites. Stimulation of prostacyclin (measured as 6-keto-$PGF_{1\alpha}$ activity) was more marked than the stimulation of PGE_2 and $PGF_{2\alpha}$ production.

We have reported previously that cultured human keratinocytes produce $PGF_{2\alpha}$ and PGE_2 material (12). These experiments confirmed those observations. Surfactant-treated cells were stimulated to produce three times as much of the two metabolites as compared to control cells. These cells did not synthesize radiolabeled metabolites eluting with standards for PGD_2, 6-keto-$PGF_{1\alpha}$, or thromboxane.

Discussion. Primary cutaneous irritancy is a common response of the skin to exogenous toxins. It represents a toxic wounding response with characteristic inflammatory and repair phases. Although this reaction can be induced by a large number of chemical and physical agents, it is fairly uniform in its appearance. Three different chemical irritants—benzalkoninum chloride, carrageenan, and probol esters—have been shown to induce production of prostaglandins in human and animal skin (5–7, 13, 14). Therefore, it appears that these membrane-derived substances are involved in mediating the inflammatory response to a variety of structurally different chemical irritants. The mechanism of action of only one group of chemical irritants has been studied in any detail, e.g., phorbol ester tumor promoters. These agents have been shown to induce inflammation, hyperplasia, and tumor promotion, through complex mechanisms which include membrane perturbation. They induce among other changes hydrolysis of arachidonic acid from membrane phospholipids and stimulate the production of prostaglandins and other arachidonate metabolites in both intact skin and cells in culture (8).

The data detailed in this report confirm the surfactant-induced release of arachidonic acid from two different types of mammalian cells in culture. Previously published studies have shown that greater than 90% of cell-associated radioactivity in the two systems outlined was incorporated into membrane phospholipids (11, 12). This suggests that ^{3}H-labeled arachidonic acid metabolites released into media in our studies were of membrane origin. A phospholipase A2 enzyme is thought to be respon-

TABLE II. SURFACTANT-INDUCED PRODUCTION OF ARACHIDONATE METABOLITES BY HUMAN KERATINOCYTES, AEOS (5×10^{-5} *M*)

Metabolite	cpm/dish		Ratio, surfactant/ control
	Control	Surfactant	
$PGF_{2\alpha}$	100	350	3.50
PGE_2	295	955	3.25
Arachidonic acid	1410	5540	3.95
Total	1805	6845	3.79

Note. Human keratinocytes were prelabeled with ^{3}H-labeled arachidonic acid and treated with AEOS (1.5×10^{-5} *M*) in growth medium without fetal calf serum or with medium alone. After 2 hr, media from surfactant-treated and control cells were extracted and the extracts were examined by HPLC for identification of ^{3}H-labeled arachidonate metabolites.

sible for hydrolysis of arachidonic acid from structural phospholipids of mammalian cells. The release induced in our studies may be due to enzyme activation or possibly to a direct detergent effect on membranes.

The time course of release within the first 15 min of treatment might suggest direct lytic effcts rather than enzyme activation. Further mechanistic studies are in progress to better define this aspect of surfactant activity.

Hydrolysis of arachidonic acid from membrane phospholipids is the first step in a very complex metabolic pathway which results in the production of a number of proinflammatory mediators, including the prostaglandins, prostacyclin, thromboxane, the monohydroxy eicosatetraenoic acids (HETEs), and the leukotrienes. The profile of metabolites produced varies greatly among cell types and species. A number of these metabolites have been shown to mediate erythema and edema production in human and animal skin. Our studies confirm surfactant-induced stimulation of the production of prostaglandins and prostacyclin (C3H-10T1/2) by mammalian cells in culture. The surfactant treatment did not stimulate production of cyclooxygenase metabolites not normally produced by control cells. In the keratinocyte experiment the stimulation of metabolites was uniform, but in the C3H-10T1/2 studies the production of prostacyclin was stimulated to a greater extent relative to the other metabolites produced. It may be that the surfactant (AEOS) induced prostacyclin synthetase enzyme activity as well as an increased hydrolysis of fatty acid from phospholipids.

It should be noted that the radioactivity associated with all cyclooxygenase products in this HPLC system accounts for only 15 to 20% of total radioactivity. Considerable radioactivity was associated with unmetabolized arachidonic acid and with products of various lipoxygenase pathways (data not shown). The identification of remaining products is now being carried out in our laboratories.

Our findings of surfactant-induced alterations of arachidonic acid metabolism with the resultant production of prostaglandins by human keratinocytes in culture suggest that the keratinocyte is the target cell for surfactant-induced primary irritancy in human skin. Utilization of that system to further define the surfactant–membrane interactions which result in mediator production should yield a better understanding of the irritant response and will also broaden our understanding of the wounding response in general.

For the present there is no consensus relative to a reliable predictive *in vitro* model for acute primary irritancy in human skin. The present method used by pharmaceutical, cosmetic, and consumer product companies of screening products for potential irritancy is limited to *in vivo* techniques using both animals and human volunteers (15, 16). These are costly and time-consuming procedures. The development of a clinically relevant *in vitro* system to screen potential irritants would certainly be humane as well as cost effective. The search for such systems in all aspects of premarketing toxicity testing is currently of prime concern to both industry and regulatory agencies.

The systems we outline here merit further investigation with a wider range of surfactants and other irritants in an effort to develop such nonwhole animal alternatives.

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