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STAPHYLOCOCCAL TOXIC EPIDERMAL NECROLYSIS: PATHOGENESIS AND STUDIES ON THE SUBCELLULAR SITE OF ACTION OF EXFOLIATIN

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An exfoliating substance elaborated by certain phage Group 2 staphylococci causes toxic epidermal necrolysis. Both in man and in the newborn mouse, intraepidermal cleavage is the predominant histologic feature following exposure to this toxin. Electron microscopic study of sequential biopsy specimens obtained from neonatal mice and from organ cultures of human skin revealed intercellular cleavage and cell separation. The extracellular nature of the exfoliative process was confirmed in several ways: (1) perfused tracers did not penetrate cells during cell separation; (2) cultured cells exposed to high doses of exfoliating fractions demonstrated no signs of injury; and (3) cleaved surfaces examined by scanning electron microscopy and surface replication demonstrated intact plasma membranes. When fractions capable of inducing exfoliation were applied to cultured keratinocytes or fibroblasts, sperm, or lymphocyte suspensions, and to human or mouse skin in vivo, they did not alter the distribution or intensity of concanavalin A binding, ruthenium red staining, pemphigus antibody binding, or HL-A surface antigens. Therefore, while the pathogenesis of staphylococcal toxic epidermal necrolysis involves intercellular cleavage, the molecular cell surface target remains unknown.

The etiology of staphylococcal toxic epidermal necrolysis (TEN) or the scalded-skin syndrome [2] is now firmly linked to a product of certain phage Group 2 organisms [3–6]. The exfoliative fraction (EF) has been isolated from culture supernatants [3–6], extensively characterized [3–5], and the disease reproduced in mice [2–6] and human volunteers [6, 7]. However, knowledge about pathogenesis is still scanty. Affected skin demonstrates intraepidermal cleavage histologically [8]. Ultrastructural studies in the newborn mouse and in man indicate that predominantly extracellular sites are attacked [6, 9], but the precise subcellular and molecular sites are unknown.

The purposes of the present study are to: (1) describe the sequential pathogenesis of the syndrome in man and the neonatal mouse on an ultrastructural level; (2) determine whether exfoliation is purely an extracellular event, and not preceded or accompanied by cytolysis; (3) describe the interaction of EF with morphologically and immunologically demonstrable surface substances in hopes of determining the molecular target on the cell surface.

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MATERIALS AND METHODS

Animals and Experimental Material

One- to three-day-old suckling Swiss or wild-type albino mice were used in all animal studies. Surgical specimens of normal, viable adult human skin, as well as neonatal mouse skin, were used for organ culture experiments (see below).

Organisms, Toxin Isolation, and Assay for Staphylococcal Exotoxins

Standard inocula (1×10^8 organisms) of two strains of phage Group 2 staphylococci, known to elaborate considerable amounts of EF and negligible alpha toxin, and control, nonphage Group 2 staphylococci were used in all these experiments (Tab. I) [10]. The culture system, cell count method, newborn mouse assay for EF, and assays for other staphylococcal toxins have been described in detail elsewhere [6–10]. Toxic epidermal necrolysis (TEN) occurred between ½ and 4 hr, depending on dilution and batch potency, after injection of EF-containing fractions. TEN was considered present when neonatal mice manifested either spontaneous skin wrinkling or a positive Nikolsky sign (skin wrinkling produced by rubbing normal-appearing skin).

EF-containing fractions were isolated and partially purified from supernatants of cultured phage Group 2 organisms as previously described [6, 10]. The potency of individual EF-containing batches was determined by subcutaneous injection of sequential dilutions into neonatal mice. For the tissue culture and immunologic experiments detailed below, material from the same

TABLE I. *TEN activity of staphylococci and sterile cell-free filtrates*

Strain	Phage type	Effective dose (live organisms)	Time (*hr*)[a]	Sterile filtrates
1	3A/3C/55/71 (Gr. II)[b]	10^5–10^9	9–10	+
2	3C/55/71 (Gr. II)	10^5–10^9	12	+
3	29/80/42E/54 (Gr. I)	No Activity 10^5–10^{10}	—	—

[a] Time when TEN was first detectable by positive Nikolsky sign.

[b] Obtained from Cross Infection Reference Laboratory, London, England.

batch adjusted to a concentration of 10 mg protein/ml normal saline (NS), was used. In general, this was about 100 times the minimum concentration necessary to induce skin wrinkling or a positive Nikolsky sign.

Tissue Culture

Pieces of human or neonatal mouse skin for organ culture were scraped free of excessive subcutaneous fat and floated, dermis side down, on culture medium. EF activity in organ culture was assayed as previously described [6]. In these experiments exposure to EF (5 mg/ml) always produced cleavage by 2 hr. Media in all cases was Eagle's Minimal Essential Media (MEM) containing 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid) buffer, 20% calf serum, and gentamycin (50 μgm/ml). Cultures were incubated at 37°C in air.

Primary and secondary cultures of guinea-pig karatinocytes and primary cultures of neonatal mouse keratinocytes were prepared using standard techniques [11]. Primary and monolayer subcultures exposed to EF or control fractions for times up to 4 days were periodically examined for evidence of toxicity on a phase microscope equipped with a heated stage and microcinematography apparatus. Toxicity manifested itself as vacuolization and rounding up of cells, followed finally by detachment of cells from the substratum.

Suspensions of guinea-pig and mouse sperm, obtained from adult animals under ether anesthesia, and fibroblast lines from dermis and lung of mice and guinea-pigs, were also used in the cytotoxicity and cytochemical experiments described below.

Tracer Injections

Injections of staphylococci, rather than EF, were used in tracer experiments, because the latent period prior to onset of TEN is constant regardless of differences in strain or inoculum size (Tab. I). Horseradish peroxidase (HRP, Sigma, 50 mg/ml normal saline), thorium dioxide (Thorotrast), or normal saline alone was injected into neonatal mice inoculated previously with cocci 6, 3, and 1 hr, and 30 min prior to onset of TEN. Other mice received tracers simultaneous with, and 30, 60, and 180 min after TEN was detectable. Biopsies were performed 20 to 30 min after tracer injection, and processed for electron microscopy (see below).

Surface-Coat Stains

Ruthenium red. The following tissues were exposed to EF and control fractions, and then treated with Luft's ruthenium red [12] prior to further processing for electron microscopy (see *Electron Microscopy* below):

a. Skin from neonatal mice biopsied at several intervals during the course of TEN.

b. Organ cultures of neonatal or human skin incubated with EF for 2 hr.

c. Primary and subcultures of guinea-pig and mouse keratinocytes, as well as several fibroblast lines incubated with EF (5 mg/ml) for 2 hr.

d. Guinea-pig and mouse sperm suspensions incubated with EF (5 mg/ml) for 2 hr. Parallel controls in all experiments were incubated with equal volumes of normal saline or with control nonphage Group 2 supernatant fractions. At termination, the persistent potency of EF-containing culture supernatants was ascertained by injection of aliquots into neonatal mice.

Monolayer cultures were gently pelleted at 1,000 *g* for 10 min, then resuspended in ruthenium red-containing fixative.

Concanavalin A plus horseradish peroxidase (HRP). Skin biopsies from neonatal mice undergoing TEN or NS-injected controls, as well as mouse and guinea-pig keratinocyte monolayer cultures treated with EF or NS alone were incubated with concanavalin A (Con-A, Miles Lab., 50 μmg/ml) followed by HRP (50 μgm/ml) according to the method of Huet and Herzberg [13]. Additional controls were incubated with Con-A or HRP alone. Pellets were prepared as above and processed for electron microscopy (see below).

Surface Replication and Scanning Electron Microscopy

In order to assess fine structural surface alterations, neonatal mice undergoing TEN were sacrificed and immediately immersed in cacodylate-buffered glutaraldehyde. While in the fixative, cleaved epidermal sheets were removed and samples of both denuded surfaces and sheets were fixed, dehydrated, and critical-point dried from CO_2 in an Aminco Instrument Co. apparatus [14]. Specimens were then either shadowed with gold–palladium and examined in a Cambridge Stereoscan scanning electron microscope (SEM) or replicated with carbon–platinum in a Balzers Freeze-Etch apparatus and examined by transmission electron microscopy.

Electron Microscopy

Skin samples and pellets were fixed in half-strength Karnovsky's fixative [15], washed with several rinses of 0.1 M cacodylate buffer, postfixed in unbuffered osmium tetroxide, dehydrated, and embedded in Epon. Peroxidase-perfused tissues and pellets were fixed and rinsed as above, sectioned at 40 to 50 μ with a tissue chopper, incubated with diaminobenzidine according to the method of Graham and Karnovsky [16], postfixed in osmium tetroxide containing potassium ferrocyanide [17], and processed as above. Ruthenium red-fixed tissues were processed in fixatives and buffers as described by Luft [12], then dehydrated and embedded as other specimens. Sections were mounted on naked copper grids and examined in a Zeiss EM 9S electron microscope.

Immunologic Techniques

Direct and indirect immunofluorescence (IF) were performed as previously described [18] using a 1:30 dilution of fluorescein-labeled antihuman IgG as conjugate (undiluted specifications: antibody 2.5 mg/ml; protein 12.4 mg/ml; antibody protein Molar ratio 0.20, antibody protein-fluorescein Molar ratio 0.063; protein-bound FITC 95.5 μgm/ml).

EF and pemphigus vulgaris. Evidence for interaction of EF with pemphigus vulgaris binding sites was sought in the following ways (Tab. II):

a. Frozen 4-μ sections of rhesus monkey esophagus were incubated with EF (5 mg/ml NS) or NS alone for 60 min at 37°C. Sections were then washed in 0.85% phosphate-buffered saline, pH 7.4, with agitation, and used as substrates for indirect IF with 2-fold dilutions of serum from a patient with pemphigus (antibody titer 1:80).

b. Three subcutaneous injections of 0.1 ml of undiluted pemphigus serum (titer 1:80) were administered every 8 hr over a 24-hr period to each of 6 neonatal mice, while 6 matched animals received the same dose of normal human serum. Animals were then challenged with sequential dilutions of EF: 2 animals in each group received undiluted EF (10 mg/ml), 1:10 dilution and 1:100 dilution, respectively. All animals were evaluated for TEN at 2 hr.

c. Frozen 4-μ sections of full-thickness skin from neonatal mice undergoing TEN, or injected with NS, were incubated with pemphigus serum for indirect IF as above.

d. Indirect IF with titered pemphigus serum was also performed on frozen 4-μ sections of a skin biopsy from a 3-year-old female inpatient in H.C. Moffitt Hospital, San Francisco, California (C.C., #6060152-1) undergoing staphylococcal TEN.

e. Specimens of normal adult breast skin, obtained at surgery, were incubated with EF for 2 hr in organ culture, at which time cleavage was occurring, then treated for indirect IF.

f. A skin lesion from a patient with active pemphigus vulgaris was biopsied, divided into two portions, and incubated with either EF (5 mg/ml) or NS for 2 hr as above, and frozen 4-μ sections were examined by direct IF for the presence of pemphigus antibody.

HL-A typing and lymphocytotoxicity studies. To determine whether EF caused lymphocytotoxicity, lymphocytes of HL-A types 1, 2, 3, 5, 7, 9, 11, 12, 13, W10, W15, and W19 were incubated with EF undiluted and at dilutions 1:2, 1:4, 1:8, 1:16, and 1:32. Lymphocytotoxicity was determined by a fluorochromatic microcytotoxicity method [19, 20].

To determine whether prior incubation of lymphocytes with EF removed cell-surface HL-A antigens, lymphocytes of specificity HL-A, 1, 3, and 12 (M.V.D.) were typed after incubation with both EF (1:2 dilution) and NS.

RESULTS

Ultrastructural Features of Early Cleavage

The fine structural features accompanying cleavage were identical in animals injected with either staphylococci or EF, and in either human or mouse skin exposed to EF in organ culture. Immediately prior to the onset of a demonstrable Ni-

TABLE II. *Summary of immunofluorescent studies*

Specimens exposed to EF, NS, or PS either in vivo or in vitro (Incubation I) were overlaid with EF, NS, or PS in frozen tissue section or organ culture (Incubation II). Specimens were then stained with fluorescein-labeled antihuman IgG and examined for intercellular fluorescence (reaction).

Tissue	First incubation	Substrate	Second incubation	Substrate	Intercellular antibody	Titer
Monkey esophagus	NS	Frozen tissue section (in vitro)	NS	Frozen tissue section	–	0
	EF		NS		–	0
	NS		PS		+	1:160
	EF		PS		+	1:160
Newborn mouse skin	NS	Liver newborn mouse (injection) (in vivo)	NS	Frozen tissue section	–	0
	EF		NS		–	0
	NS		PS		–	0
	EF		PS		–	0
Normal human skin	NS	Organ culture (in vitro)	NS	Frozen tissue section	–	0
	EF		NS		–	0
	NS		PS		+	1:160
	EF		PS		+	1:160
TEN staph skin	EF	Patient with TEN (in vivo)	NS	Frozen tissue section	–	0
	EF		PS		+	1:160
Pemphigus skin	PS	Patient with pemphigus (in vivo)	NS	Frozen tissue section	+	NA
	PS		EF		+	NA
Pemphigus skin	PS	Patient with pemphigus (in vivo)	NS	Organ culture	+	NA
	PS		EF		+	NA

Key: NS = normal saline; EF = exfoliative fraction; PS = pemphigus serum; NA = not applicable (direct immunofluorescence).

kolsky phenomenon, keratinocytes on thin section appeared completely normal, and could not be differentiated from those of control skin samples. Furthermore, even when a Nikolsky sign could already be elicited, keratinocytes appeared normal. At the time that early, spontaneous wrinkling occurred, two fine structural patterns emerged: focal interdesmosomal dilatations formed in some regions (Fig. 1*a*), while in other samples separation occurred simultaneously along both desmosomal and interdesmosomal surfaces (Fig. 1*b*). Here too, keratinocytes appeared normal. At later stages, acantholytic keratinocytes floated freely in cleavage spaces; but even in advanced lesions, cells bordering cleavage cavities and acantholytic cells appeared normal. Cells displayed villous-like projections topped by split half-desmosomes which remained associated with tonofilaments (Fig. 1*a*, *b*).

Features of Cleaved Intraepidermal Surfaces

Surface replicas of cleaved surfaces revealed a smooth surface coat studded by regular knob-like protrusions (Fig. 2*a*). In some regions, the surface coat was interrupted, exposing a still smoother membrane bearing regular 6-nm to 10-nm particles, presumed to represent the outer surface of the trilaminar membrane (Fig. 2*b*). In no case did we observe cracks, holes, or other irregularities of this surface, which might represent damage to the cell membrane.

On SEM, a similar pattern was seen: a very homogeneous surface film covered large portions of the denuded surfaces (Fig. 3*a*), but in some cases this "coat" or film detached or curled to reveal an underlying surface with a somewhat more complicated topography (Fig. 3*b*). Again, no "breaks" or defects suggesting cytolysis were seen in this lower plane.

Ultrastructure of Tracer-Perfused Tissues

After intracutaneous injections of HRP or Thorotrast, tracer percolated freely upward from the dermis filling the intercellular spaces. Again the fine structure of control specimens and early TEN specimens (positive Nikolsky sign without spontaneous wrinkling) were identical, i.e., tracer was confined to the intercellular space and keratinocytes were unaltered (Fig. 4*a*). During both early (Fig. 4*a*, *b*) and late (Fig. 4*b*, *c*) cleavage, tracer remained localized to the intercellular space. Cells bordering cavities, as well as free-floating, acantholytic cells, did not admit tracer.

Cytologic Features of Cultured Monolayers Exposed to EF

In Figures 5*a* and 5*b* are compared the microscopic appearance of control (NS) and EF-treated mouse keratinocyte cultures, respectively. After a 4-hr exposure to EF the appearance of cells is comparable to controls. Trypan blue exclusion studies revealed no differences between the viability of EF and control cultures. Culture supernatants retained their full capacity to produce TEN in neonatal mice, and comparable concentrations of EF produced cleavage of human and neonatal mouse skin in organ culture.

Surface-Coat Staining of EF-Treated and Control Cells

In order to detect EF removal of stainable cell surface polysaccharides, treated cells were stained with ruthenium red (acidic mucopolysaccharides) and Con-A (neutral polysaccharides).

Ruthenium red. Both control mice and EF-injected animals demonstrated faint ruthenium red surface-coat staining. In specimens where early separation was occurring, ruthenium red continued to be deposited on adjacent surfaces (Fig. 6*a*). Monolayer cultures of keratinocytes and fibroblasts, as well as sperm suspensions stained equally intensely with ruthenium red, whether exposed to EF or control solutions (Fig. 6*b*, *c*, *d*).

Con-A plus HRP. The epidermal intercellular spaces of neonatal mice undergoing TEN and those of NS-injected controls stained similarly with Con-A (Fig. 7). Furthermore, cultures of murine keratinocytes incubate with EF for 2 hr demonstrated normal staining with Con-A. On the other hand, in cultures incubated with either Con-A alone of HRP alone, surface-coating staining was absent or negligible.

Immunologic Studies (Table II)

Pemphigus vulgaris. Pemphigus vulgaris is a bullous disease characterized by intraepidermal, intercellular separation accompanied by the fixation of specific antibody and complement within the intercellular space. In order to detect whether EF interacts with the same cell-surface components that bind pemphigus antibodies, the following immunologic studies were performed: Indirect IF of sections of monkey esophagus, normal human skin, and newborn mouse skin, incubated first with EF then with pemphigus antibody, appeared indistinguishable from control skin sections that had not been incubated with EF. Neonatal mouse skin did not bind pemphigus antibody from either of two sera from patients with pemphigus, and fluorescence could not be demonstrated. When indirect IF tests were performed on the patient with staphylococcal TEN, a normal pattern of pemphigus antibody binding was observed; indirect IF of both the patient's and control sections was positive to a titer of 1:160.

Direct IF of active pemphigus skin lesions treated with EF in organ culture or on sections revealed persistent, normal intercellular fluorescence. Incubation of sections of skin from the patient with pemphigus vegetans likewise showed no qualitative diminution of intercellular staining after incubation with EF.

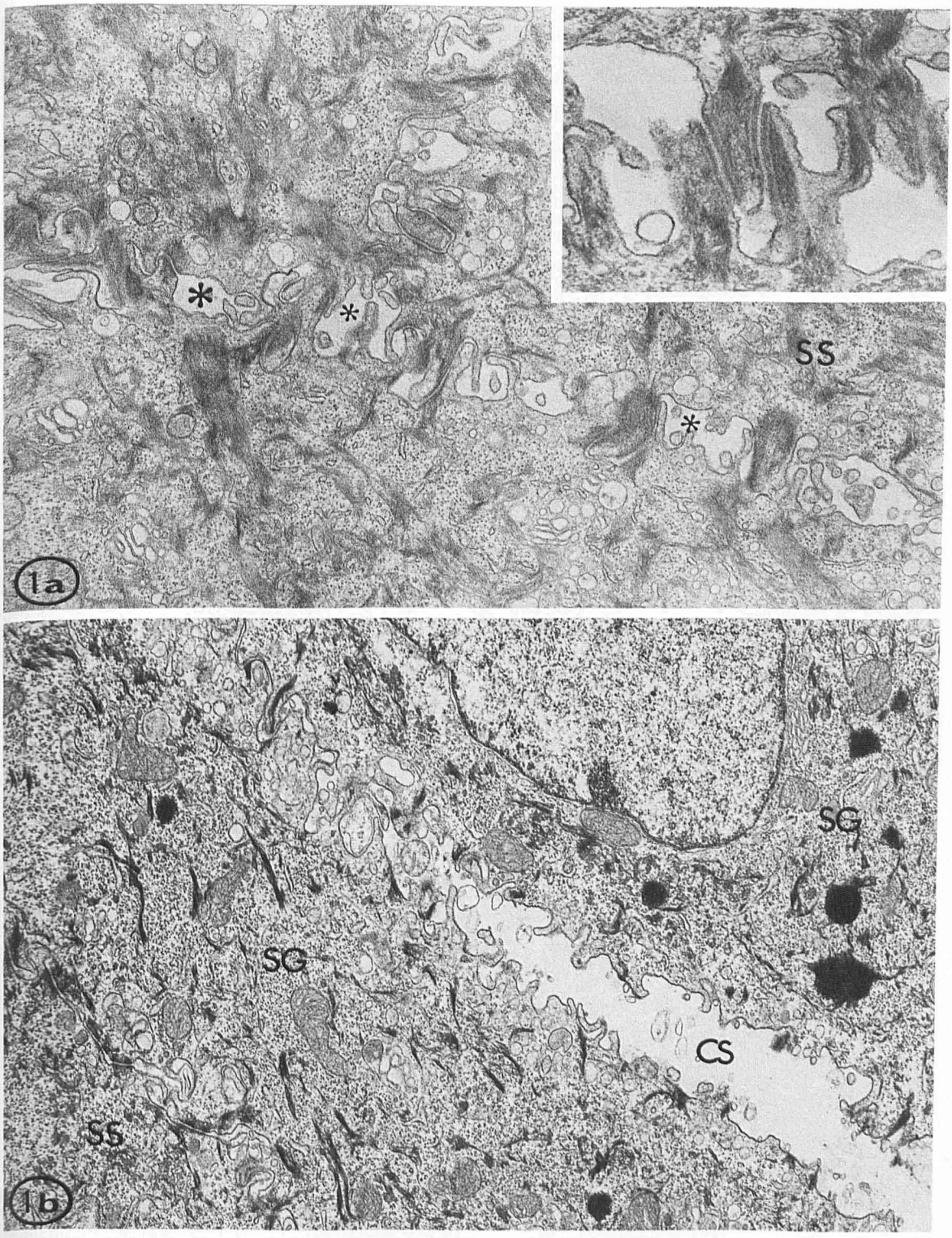
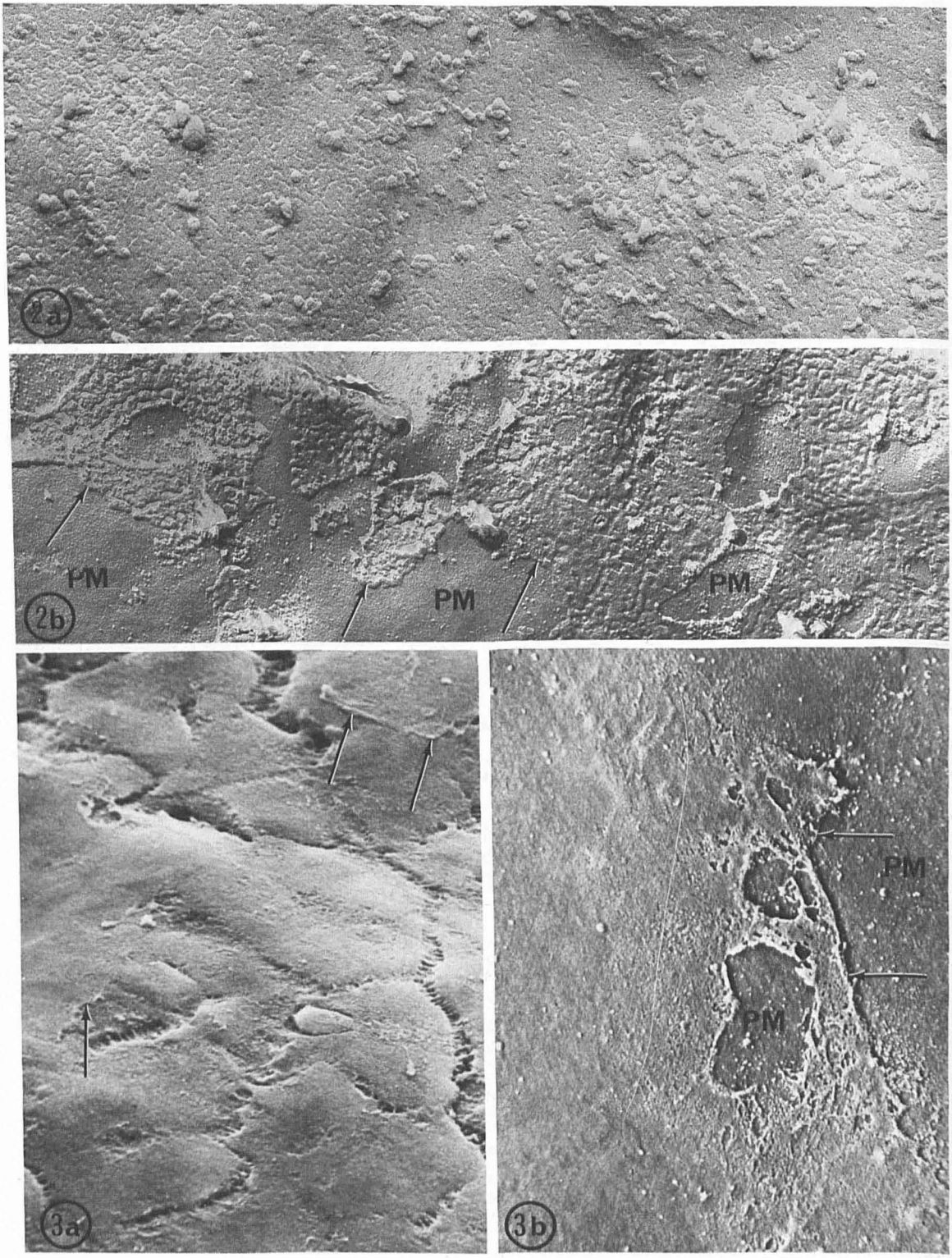


FIG. 1. Patterns of early cell separation following injection of exfoliatin. In both 1*a* and 1*b* the cells appear intact and uninjured. Early separation may involve interdesmosomal ballooning (*) (compare 1*a* *insert* and Fig. 6*a*) or simultaneous cleavage of both desmosomes and interdesmosomal regions (1*b*) at the spinous (*SS*)–granular layer (*SG*) interface. The end result is the same—acantholysis without evident cell disruption, and the formation of a cleavage space (*CS*) 1*a* × 16,000, *insert* × 35,000; 1*b* × 9,500).



FIGS. 2 and 3. Surface replicas of exfoliatin-separated cell surfaces viewed by transmission (2*a*, 2*b*) and scanning (3*a*, 3*b*) electron microscopy. In both cases a surface coat or film covers all (2*a*) or much of the exposed surfaces. Where the coat is detached (*arrows*) a subjacent layer, presumably the outer leaflet of the plasma membrane (*PM*) is revealed. No defects or holes can be seen in this layer. In some regions adjacent cells in early stages of detachment appear to be connected by attenuated cell processes (*double arrows*), probably linked by desmosomes (2*a* × 85,000, 2*b* × 40,000; 3*a* × 1,050, 3*b* × 1,850).

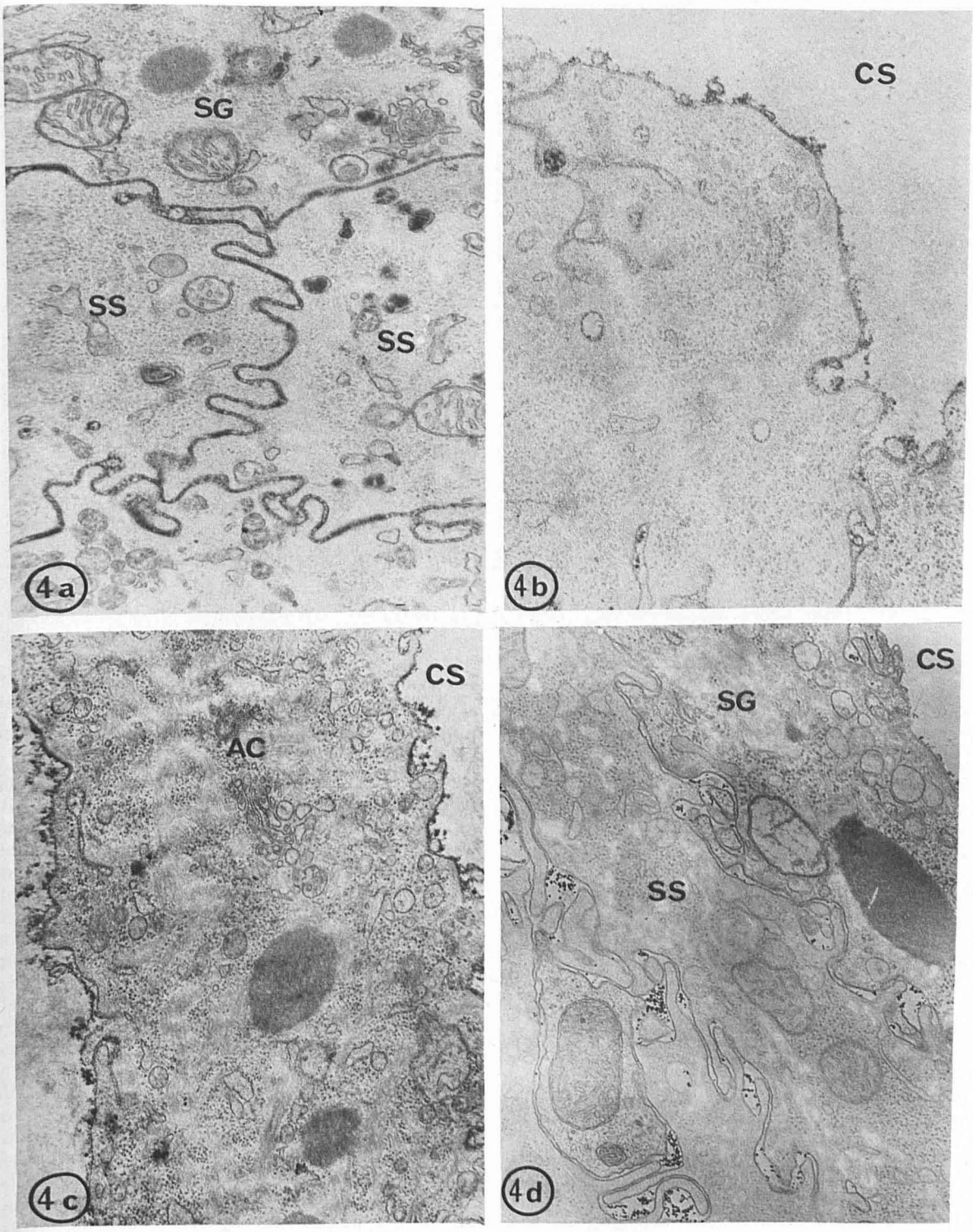


FIG. 4. Tracer-perfused neonatal mouse skin in various stages of exfoliation. Figures 4*a*–4*c* depict various stages of cleavage in horseradish peroxidase (HRP)-perfused tissues. At the time when spontaneous wrinkling had not occurred, but a positive Nikolsky sign could be elicited (4*a*), no leakage of tracer into granular (*SG*) and spinous (*SS*) cells occurred, and cells appear completely unperturbed. During early, spontaneous cleavage (4*b*) and late cleavage (4*c*), HRP still does not enter either cell's lining cleavage space (*CS*) or free-floating acantholytic cells (*AC*) (4*c*). Figure 4*d* depicts intermediate cleavage in Thorotrast-perfused epidermis. Again, tracer does not enter cells in affected regions (4*a* × 10,000, 4*b* × 22,000, 4*c* × 16,000, 4*d* × 12,000).

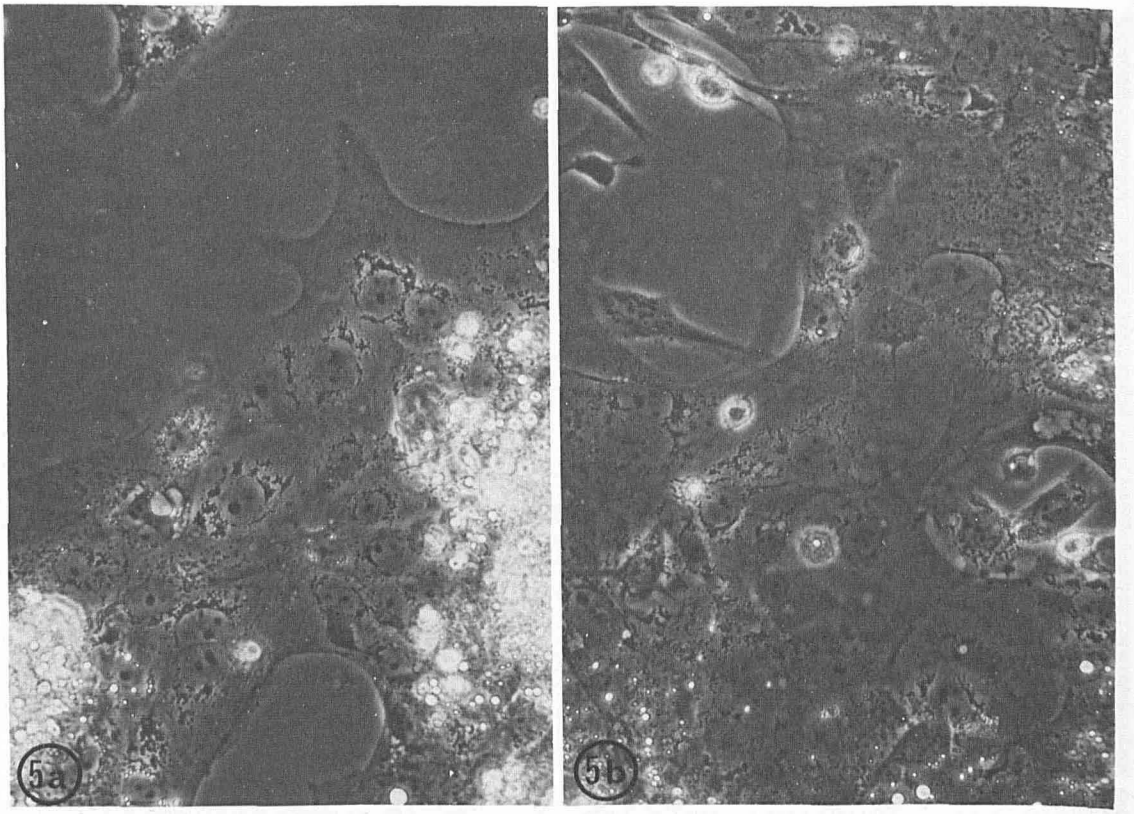


FIG. 5. Primary culture of mouse keratinocytes exposed to either exfoliatin-containing fractions (EF) (5*a*) or untreated controls (5*b*). After 4 hr there is no apparent difference in cytomorphology of either culture. EF-containing supernatants retained capacity to cause exfoliation in neonatal mice throughout incubation period (5*a* and *b* × 1,000).

HL-A and Lymphocytotoxicity Studies

EF was not cytotoxic to lymphocytes of specificities HL-A 1, 2, 3, 5, 7, 9, 11, 13, W10, W15, and W19 when diluted at least 1:2 in fresh, heat-inactivated serum, producing no detectable decrease in lymphocytotoxicity titer as compared to saline preincubated controls. At the conclusion of experiments, TEN was produced in neonatal mice injected with the most dilute solutions. Lymphocytes of specificities HL-A 1, 3, and 12 typed normally after prior incubation with EF diluted 1:2 in fresh, heat-inactivated serum.

DISCUSSION

Evidence for an Extracellular Site of Action of Staphylococcal Exfoliatin.

Histologic features. The histologic picture which distinguishes all clinical forms of staphylococcal TEN (i.e., bullous impetigo, generalized TEN, TEN in the experimental mouse model) from other types of TEN is the presence of a subgranular, intraepidermal cleavage plane [8,21]. Furthermore, while other forms of TEN are histologically characterized by widespread epidermal necrosis, the cells adjoining early cleavage planes in staphylococcal TEN appear normal.

Ultrastructural studies—evidence from morphology. In an ultrastructural study of newborn mice undergoing experimental staphylococcal TEN, Lillibridge et al first noted that the disease process involves cell separation without significant cytolysis [9]. They also observed fine structural alterations in the vicinity of desmosomes preceding cell separation, and therefore considered desmosomal regions the primary site of EF attack. In a previous study of experimental staphylococcal TEN in man and the mouse [6,10], we noted that both in in vivo and in organ culture, the exfoliatin caused cell separation without evident cytolysis. In this study we have studied the pathogenesis of the syndrome in neonatal mice in greater detail. During early separation desmosomes often clung together, while interdesmosomal regions ballooned (e.g., Figs. 1*a*, 6*a*), but in other sites separation appeared to occur simultaneously along entire adjacent cell surfaces (Fig. 1*b*). Thus, desmosomes do not appear to be the primary target of EF [9]. Instead, substance(s) along interdesmosomal regions or uniformly present along the entire surface appears to be attacked.

The basis for the midepidermal site of action remains enigmatic. No unique epidermal structures are present or forming at this interface. Lamellar bodies (Odland bodies, membrane-coating granules) are extruded apical to this site, and their contents may prevent access of EF to more

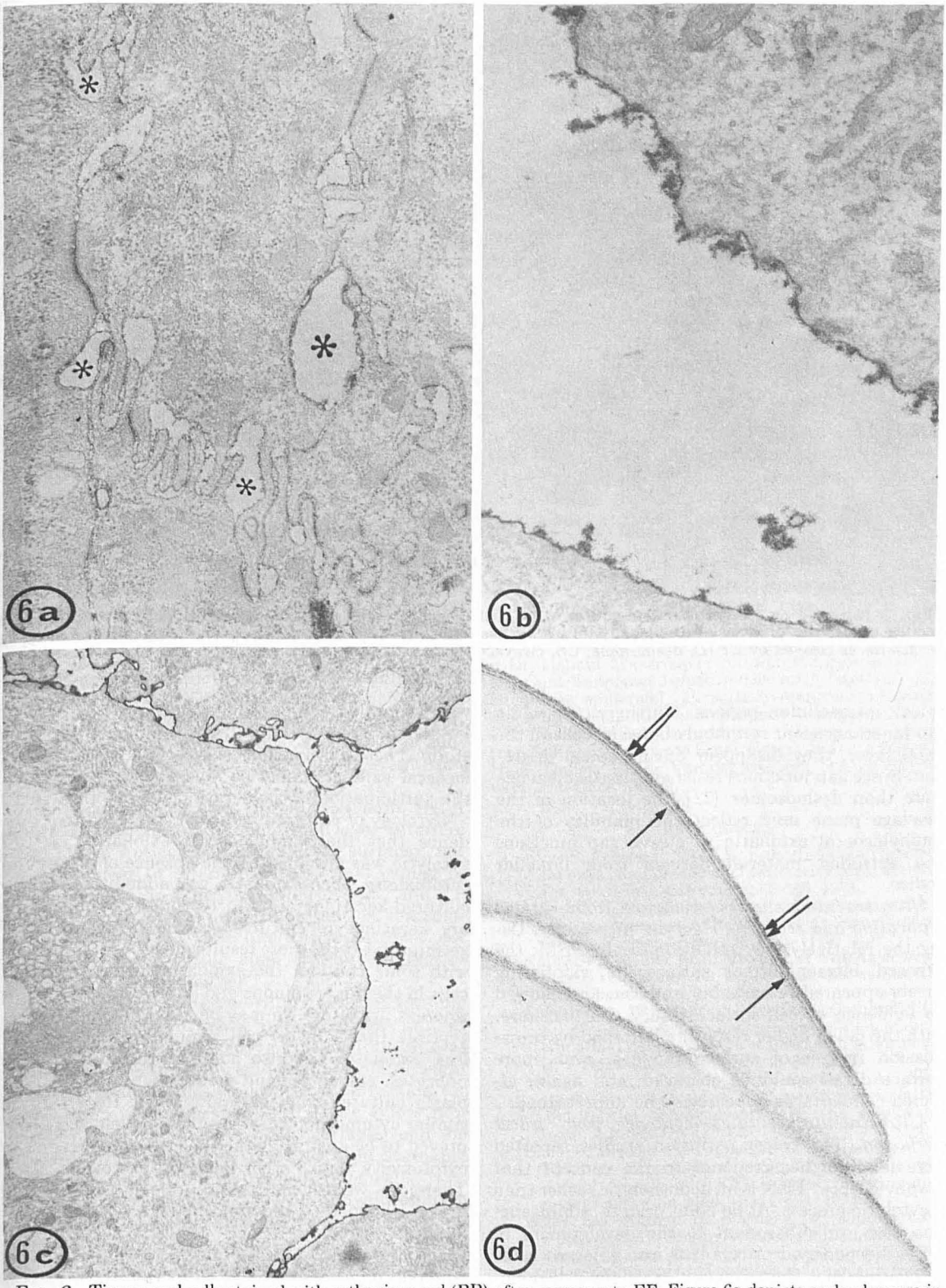


FIG. 6. Tissues and cells stained with ruthenium red (RR) after exposure to EF. Figure 6*a* depicts early cleavage in neonatal mouse skin. Mouse skin in vivo generally stains weakly, but staining persists in regions of early separation (*). Figures 6*b* and 6*c* illustrate persistent RR staining of mouse keratinocytes (6*b*) and fibroblasts (6*c*) after exposure to EF, while Fig. 6*d* demonstrates staining of surface coat of both acrosomal (*arrows*) and plasma membrane (*double arrows*) of guinea-pig sperm after EF treatment (6*a* × 8,000, 6*b* × 36,000, 6*c* × 19,000, 6*d* × 58,000).

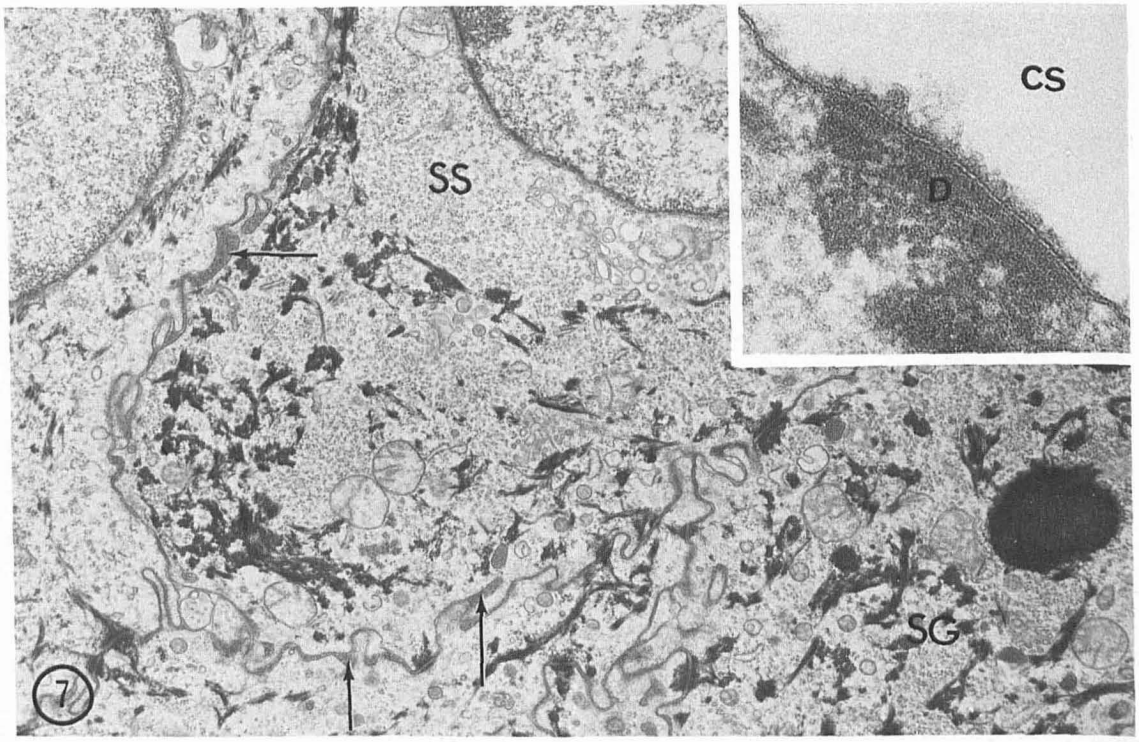


FIG. 7. Neonatal mouse skin exposed to Con A + HRP. Note intercellular staining (*arrows*) of control cells at junction of stratum granulosum (*SG*) and stratum spinosum (*SS*). Insert illustrates persistence of Con A staining along surfaces cleaved by EF (*D*, desmosome; CS, cleavage space) (× 6,000, *insert* × 22,500).

apical intercellular regions. Furthermore, while gap junctions occur commonly between cells in the basal layer, they disappear during apical migration. Since gap junctions resist enzymatic cleavage more than desmosomes [22], the location of the cleavage plane may reflect the inability of the staphylococcal exfoliatin to cleave gap junctions and extruded material derived from lamellar bodies.

Ultrastructural studies—evidence from surface replication and scanning electron microscopy. Using the relatively low resolution of the SEM, the exposed intraepidermal surfaces of exfoliating sheets appeared remarkably uniform, and showed no evidence of cell-surface injury. Furthermore, with the much higher resolution afforded by transmission images of surface replicas, even more surface detail could be observed, and again, affected cell surfaces appeared to be unperturbed.

Ultrastructural studies—evidence from tracer perfusion. The tracer perfusion studies reported here lend further credence to the concept that staphylococcal TEN is an acantholytic rather than a cytolytic process. At no point prior to, simultaneous with, or subsequent to the development of TEN did perfused tracers leak into adjacent cells, indicating that the permeability of the cell membrane was not affected, at least with regard to water-soluble tracer molecules of the size (molecular weight greater than 40,000) employed in this study. These observations suggest that the staphylococcal exfoliatin may be able to cleave without the participation of the keratinocyte cytoplasm.

Cytology of cultured keratinocytes. Further evidence that the staphylococcal exfoliatin is not cytolytic was provided by the absence of observed cytotoxicity when exfoliatin was added to lines of cultured keratinocytes. Since primary and secondary keratinocyte cultures presumably consist of germinative cells, these results must be interpreted with some caution: the exfoliatin in vivo cleaves cells in the upper spinous and lower granular layer, whereas in culture we may be observing a nonsusceptible (basal layer) cell population. Nevertheless, exfoliatin was also nontoxic to human lymphocytes, guinea-pig and mouse sperm, and fibroblasts cultured from several tissues. Recently, a similar cytomorphologic assay was developed, and proved to be a valid method for the assessment of cytotoxicity of two other bacterial exotoxins [23]. Therefore, we feel that these cytologic studies add major evidence to the concept of a nontoxic exfoliatin.

An essentially extracellular site of action of staphylococcal exfoliatin is supported by: (1) fine structural morphology, (2) absence of leakage of

intercellular tracers into cells, (3) unaltered cell surfaces of toxin-separated cells on SEM and surface replication, and (4) absence of demonstrable cytotoxicity to cultured keratinocytes and lymphocytes.

Search for Specific Target Substances on the Cell Surface

Immunologic markers—interaction with pemphigus antibody binding sites. Since acantholysis of epidermal cells is a prominent feature of the bullous disease, pemphigus, we sought evidence of similar pathophysiologic mechanisms for pemphigus and staphylococcal TEN. Sera of patients with pemphigus contain antibodies which attach to the intercellular space of stratified squamous epithelia [24], including surfaces attacked by EF. Our hypothesis was that the cell membrane receptor for the staphylococcal exfoliatin and the pemphigus antigen might be related or identical. This interest was spurred by recent evidence that de novo deposition of pemphigus antibody in intercellular sites may be the actual cause of the acantholysis in pemphigus [25,26], and not merely a concomitant event. However, the studies reported here failed to demonstrate blockade of pemphigus antibody staining by EF on indirect and direct IF. Furthermore, passive infusion of pemphigus antibody into neonatal mice, as well as in vivo binding of pemphigus antibody to skin in a patient with pemphigus, did not interfere with subsequent in vivo or in vitro production of TEN by exfoliatin. It appears that: (1) EF does not alter pemphigus antigen, or sterically or otherwise interfere with pemphigus antibody binding, and (2) the substance(s) attacked by EF is probably different from pemphigus antigen.

Immunologic markers—interaction with HL-A antigens. Exfoliatin did not remove or interfere with HL-A antigens on lymphocyte surfaces, nor did it appear to interfere with normal typing of cells bearing the HL-A specificities 1, 3, and 12. Since HL-A antigens are also present on keratinocyte surfaces [27], it is likely that EF does not attack HL-A antigenic sites of the specificities tested in epidermis either.

Cytochemical markers—ruthenium red staining in vivo and in vitro. While anionic charges present on cell surfaces may play a role in intercellular repulsion [28], the sialic acid residues contained in acid mucosaccharides could also be important for aggregation and adhesion [29]. These acidic mucosubstances present on cell surfaces can be stained with ruthenium red [12], and stainable material is removed by treatment of cells [13], including keratinocytes [30] with hyaluronidase and neuraminidase. Accordingly, an analogous effect was sought for exfoliatin. However, tissues and cells treated with EF both in vivo and in vitro demonstrated no diminution in ruthenium red staining, while neuraminidase and hyaluronidase remove stainable material [29], indicating that EF probably does not remove stainable surface acid mucopolysaccharides in significant quantities.

Cytochemical markers—concanavalin A plus HRP staining in vivo and in vitro. Neutral oligosaccharides on cell surfaces play an important role in cell agglutination (reviewed in [31]). The lectin, concanavalin A (Con-A), displays a striking affinity for the α-D-methyl glucopyranoside moieties of such neutral sugars [31], and lectin binding sites can be visualized ultrastructurally by staining bound Con-A with horseradish peroxidase [13]. Since we observed no diminution in Con-A–HRP staining of murine keratinocytes treated with EF fractions both in vivo and in vitro, we believe that cell surface neutral sugars are not the primary target of the staphylococcal exfoliatin.

The staphylococcal exfoliatin is remarkably nontoxic to keratinocytes, producing the clinical features of the "scalded-skin syndrome" by a purely extracellular attack on an as yet unidentified substance(s). Yet, utilizing several cytochemical and immunologic markers, we have been unable to demonstrate removal of morphologically detectable surface-coat substances from keratinocytes. Therefore, the exfoliatin may attack other, as yet unidentified, surface substances, or it may be able to cleave keratinocytes through removal of small, morphologically undetectable quantities of the target molecules that we have studied.

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