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Cutaneous Exposure to Bis-(2-chloroethyl)sulfide Results in Neutrophil Infiltration and Increased Solubility of 180,000 M_r Subepidermal Collagens

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ABSTRACT. Exposure to bis-(2-chloroethyl)sulfide (BCES; "sulfur mustard") causes delayed formation of slowly healing skin blisters. Although the histopathology of BCES injury is well characterized [reviewed in Smith *et al.*, *J Am Acad Dermatol* **32**: 767–776, 1995], little is known of the cutaneous toxicity at the molecular level. To identify biological markers of exposure, epidermal and subepidermal extracts were prepared from 48 individual hairless guinea pigs (HGP) at successive 3-hr intervals following exposure to BCES vapor, and compared using gel electrophoresis, and lectin- and antisera-binding. Inflammation was assessed by measuring edema and myeloperoxidase activity. Edema reached peak levels at 15–18 hr and remained elevated above controls at 24 hr. Recruitment of neutrophils, deduced from increased myeloperoxidase, occurred as early as 3 hr after BCES exposure with maximum infiltration at 6–12 hr. Binding of concanavalin-A lectin revealed increased amounts, relative to contralateral control sites, of two approximately 180,000 M_r polypeptides in subepidermal protein extracts from the BCES-exposed skin obtained ≥12 hr after exposure. This alteration was not found in epidermal protein extracts prepared from the same animals. Based upon the determined amino acid compositions, both polypeptides had significant collagenous triple helical content (>75%). They could be distinguished immunologically from collagen types I, III, and IV by using polyclonal antisera. We conclude that exposure of HGP skin to BCES results in an early neutrophil infiltration that precedes epidermal-dermal separation and selective alterations of the subepidermal extracellular matrix. BIOCHEM PHARMACOL 53;10: 1405–1412, 1997. Published by Elsevier Science Inc.

KEY WORDS. sulfur mustard; hairless guinea pig; myeloperoxidase; collagen

Skin, respiratory tract, or eyes exposed to BCES† undergo a variable latent phase followed by a classical inflammatory sequela that culminates with separation of basal epithelial cells from the underlying dermis or stromal tissue at the basement membrane zone [reviewed in Refs. 1–5]. Lesions are slow to develop and especially recalcitrant to healing [6–9]. Histopathology of BCES exposure has been studied in several animal species and found to resemble injury from other DNA-damaging agents, including ultraviolet radiation [10–12; reviewed in Ref. 13]. The cause(s) of vesication and effective medical countermeasures, however, remain unknown.

The euthymic HGP provides a useful skin model that possesses a multiple-cell-layer epidermis and pharmacological similarities to humans [13, 14]. Previous studies of the HGP have documented the toxicity of BCES with respect to erythema, histopathology, and electron microscopical pathology [15–20]. Ultrastructural analyses of HGP skin exposed to BCES have revealed a tissue cleavage plane that is localized to the basal cell basement membrane zone, proximal to immunohistochemical staining for laminins and collagen type IV [19–21]. The subepidermal separation found in the HGP is comparable to observations of human skin exposed to BCES [reviewed in Ref. 5], as well as to human skin explants exposed to the vesicant *ex vivo* [22]. Our goal was to augment qualitative histopathology obtained in the HGP model by identification of specific subepidermal molecules that might serve as biological markers for BCES cutaneous toxicity.

MATERIALS AND METHODS

BCES Challenge and Tissue Collection

Skin samples were from 48 male HGP (*Cavia porcellus*; Crl:IAF/HA(hr/hr)BR Vaf/PlusU/U). Animals were quarantined after arrival from Charles River Laboratories (Wilmington, MA), determined to be healthy, and maintained in an accredited animal care and use facility. After anesthetization with 30 mg/kg ketamine-HCl (Vetalar; Parke-Davis, Morris Plains, NJ) mixed with 6 mg/kg xylazine (Rompun; Mobay Corp., Shawnee, KS), animals were

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† *Abbreviations*: βME, 2-mercaptoethanol; BCES, bis-(2-chloroethyl)sulfide, "sulfur mustard"; con-A, concanavalin-A; HGP, hairless guinea pig; MPO, myeloperoxidase; PVDF, polyvinylidene difluoride; and %T, total acrylamide + bisacrylamide.

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weighed, and the air temperature of the laboratory hood was measured.

Some of the 48 animals were placed in one "naive" control group (8 animals) that was not exposed to agent; the other animals were divided into eight groups exposed to BCES with skin collection at different post-exposure observation times of 3, 6, 9, 12, 15, 18, 21, and 24 hr (5 animals/group, total of 40).

All animal exposures were conducted on the dorsal-lumbar region with saturated vapor (98% purity; approximately 0.77 g/m^3 for a time of 7 min) using an established vapor cup (10 mm diameter) method that is known to produce consistent degrees of skin injury [17, 18]. From each exposed animal, we analyzed the polypeptide composition of three to five BCES sites and two sham control sites (received vapor cup with no BCES). Separate skin sites (exposed and sham controls) from each animal were used for MPO studies. Remaining skin punches from each of the 48 animals were processed for routine histopathological evaluation.

Skin Extracts

Euthanasia at the appropriate time was by an inhalation overdose of halothane. Within 5 min after death, skin was excised carefully, the subcutaneous hypodermis was removed, and 8 mm diameter skin punches were placed in 500 μL of high salt buffer consisting of 1.0 M NaCl, 0.15 M Tris (pH 9.3) with a mixture of protease inhibitors [30 μg/mL (4-amidinophenyl)-methanesulfonylfluoride, 0.2 mg/mL EDTA, 300 μg/mL phosphoramidon, 0.5 μg/mL leupeptin, 50 μg/mL antipain-dihydrochloride and 0.5 mg/mL E-64, a cysteine protease inhibitor; all from Boehringer Mannheim Biochemicals, Indianapolis, IN). Samples were incubated in this buffer at 4° for 72–80 hr.

The epidermis and the dermis were separated easily after the high salt incubation by employing lateral traction under a stereo dissecting microscope fitted with cold fiber optic illumination. Major epidermal proteins were solubilized by heating the epidermal sheets at 100° for 5 min in 8 M urea/0.3 M βME (pH 9.3). Preparation of subepidermal extracts was performed essentially as described [23–25]. Superficial dermis was extracted with 8 M urea/0.3 M βME at 23° for 90 min with gentle rocking.

Individual extracts were centrifuged (30 min at 16,000 *g*), and the supernatant was brought to 1% (w/v) SDS/0.01% (w/v) bromophenol blue, heated to 100° for 5 min, and stored at −88°. An additional 3% (v/v) βME was added, and the samples were heated to 100° again for 1 min immediately prior to SDS–PAGE.

Polypeptide Separation by SDS–PAGE

Total protein of epidermal and subepidermal extracts was measured [26] relative to BSA (Calbiochem, La Jolla, CA). SDS-polypeptide complexes were separated by SDS–PAGE (4%T stacking/6.5%T separating gel) using the Laemmli [27] buffer system at 3.5°.

Con-A Lectin Binding

Separated polypeptides were electrophoretically transferred to PVDF microporous membrane (Millipore Corp., Bedford, MA) and incubated overnight in 3% (w/v) BSA. Transfer conditions were optimized initially by staining with amido black. Lectin binding was carried out as described [28, 29] using con-A (25 μg/mL) followed by peroxidase-conjugated anti-immunoglobulin (1:500; DAKO, Carpinteria, CA) and visualized with diaminobenzidine-HCl and hydrogen peroxide.

Antisera Binding

Several rabbit polyclonal antisera to rodent collagens were tested for binding to the HGP skin subepidermal extracts, including: anti-mouse skin collagen type I; anti-rat skin collagen type I; anti-rat skin collagen type III, and anti-mouse collagen type IV (isolated from Englebreth-Holm-Swarm sarcoma). The polyclonal antisera were obtained from Chemicon Int. (Temecula, CA) and used at 1:1000 dilution. Methods paralleled those used in lectin studies except that the PVDF was blocked in 5% (v/v) normal goat serum. Primary antibody binding was detected with peroxidase-conjugated secondary anti-rabbit immunoglobulin (1:1000 dilution; DAKO). Control experiments included replacement of primary antibodies with non-immune serum.

Image Analysis

Digitalized images of gels or stained PVDF sheets were acquired with a high-speed, low-light camera (Megaplus 1.4, Kodak, Rochester, NY) connected to a Sparc-2 workstation (Sun Microsystems Inc., Mountainview, CA). The camera was calibrated with 21-step graded standards of known reflectance and density. The M_r values and integrated optical densities of stained polypeptides were calculated using Investigator and Whole Band Analysis software packages (Bio-Image, Ann Arbor, MI).

Amino Acid Composition

Electrophoretic transfer was conducted using ϵ-amino caproic acid instead of glycine. Pieces of PVDF containing the 180,000 M_r polypeptides of interest were cut from amido black-stained lanes, washed extensively with water, and subjected to acid hydrolysis *in situ* [30]. Recovered amino acids were derivatized with phenylisothiocyanate and analyzed relative to phenylthiocarbamyl-amino acid standards (Pierce, Rockford, IL) by reverse-phase HPLC separation with detection at 254 nm as described [31]. Adjacent pieces of "blank" PVDF were analyzed separately to determine background amino acid levels [30]. Composition data for both polypeptides were compared with that of proteins contained in the SwissProt (5/1995) database using the PROPSEARCH algorithm [32].

MPO Assay

Separate, full thickness skin punches from each animal were weighed and homogenized in 0.5% hexadecyltrimethyl-ammonium bromide in 50 mM potassium phosphate (pH 6.0) using a Polytron (model PT1200, Kinematica, Littau, Switzerland) at 12,000 rpm for 1 min at 2–4°. The homogenate from each sample was centrifuged (20 min at 16,000 g), and the supernatant was assayed for MPO activity by monitoring the change in absorbance at 450 nm in the presence of 0.167 mg/mL 3,3′-dimethoxybenzidine dihydrochloride, and hydrogen peroxide in 50 mM potassium phosphate (pH 6.0), 37° [33].

Statistical Methods

Values for total MPO activity and tissue wet weight were expressed as the mean ± SEM for each post-exposure time interval. Significant increases in BCES-treated or contralateral control sites above normal, naive HGP skin were determined by calculating and comparing the 95% confidence intervals as described [34].

RESULTS

Skin Variables

Animal weight was 350 ± 30 g (mean ± SEM). The hood temperature during exposure was 17.4 ± 0.5°. The total protein concentration was 0.7 ± 0.2 mg/mL for subepidermal extracts and 1.8 ± 0.1 mg/mL for epidermal extracts.

Skin Edema

Significant elevation of tissue wet weight above the control site did not occur until 6 hr after BCES exposure, with the maximum edema response observed at 15–18 hr (Fig. 1). Reduced standard error was observed in wet weight values at peak edema time points, suggesting saturation. Edema apparently was not resolved at 24 hr after exposure because BCES-exposed sites were still significantly heavier than control sites (Fig. 1).

Neutrophil Infiltration

Vapor exposure to BCES increased cutaneous MPO activity above values obtained from naive, unexposed HGP skin at the earliest post-exposure time observed (3 hr; Fig. 2). Contralateral control sites from exposed animals also showed increased MPO levels, consistent with a diffuse infiltration of neutrophils to the site of skin injury. However, sites exposed directly to BCES showed significantly greater activity than contralateral control sites by 6 hr after exposure. Maximal neutrophil influx was observed between 6 and 12 hr after BCES exposure (Fig. 2). The MPO response preceded epidermal-dermal separation; the latter occurred at 15–18 hr after an identical BCES exposure in the HGP model [16, 17].

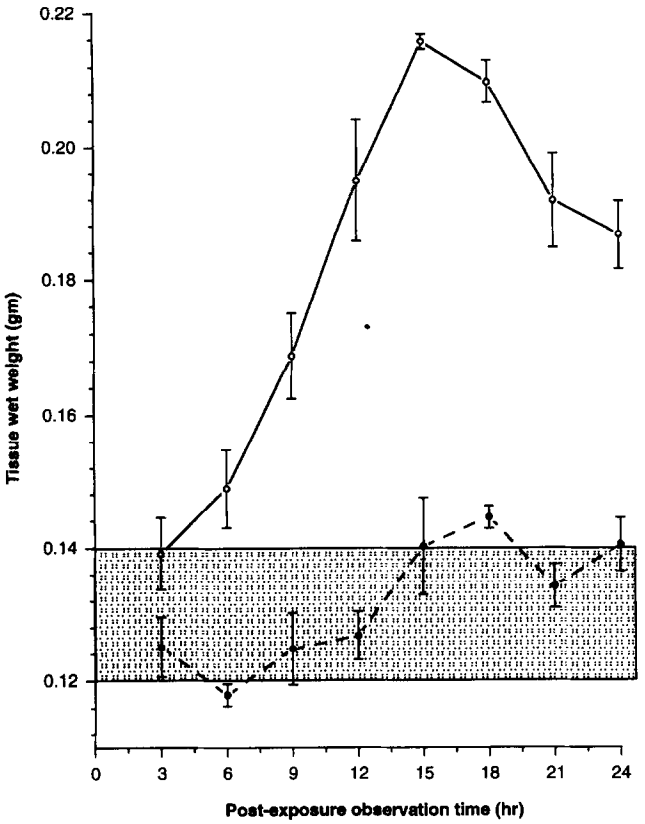

FIG. 1. Effect of BCES exposure on HGP skin edema. Each time point is the mean ± SEM of single skin samples from four animals. BCES exposure (open circles, solid line) increases the tissue wet weight significantly above contralateral control sites (closed circles, broken line) from the same animals. For reference, the shaded box shows the 95% confidence interval for wet weight values of 15 skin samples obtained from a total of 8 naive control animals (no BCES exposure).

Dissection of Epidermis from Dermis

Upon inspection by stereoscopic 40-fold magnification, skin exposed to BCES demonstrated marked erythema, occasional fluid-filled microvesicles, and excessive dilation of subepidermal vessels compared with control sites. Histopathology results were qualitatively comparable to those reported previously in the HGP following identical BCES vapor exposures [17, 18].

The high salt split method employed, also called "artificial epidermolysis," removes the epithelium through the lamina lucida [24, 25]. The HGP epidermis retracts easily as a single sheet from the dermis, facilitating a fractionation of the extractable, subepidermal proteins from those components that remain associated with the epidermis.

SDS–PAGE

The extraction method used has been reported to enrich for extracellular matrix molecules of the basement membrane zone and superficial dermis [23–25]. Not surprisingly, therefore, the primary protein constituents of our HGP subepidermal extracts were collagens.

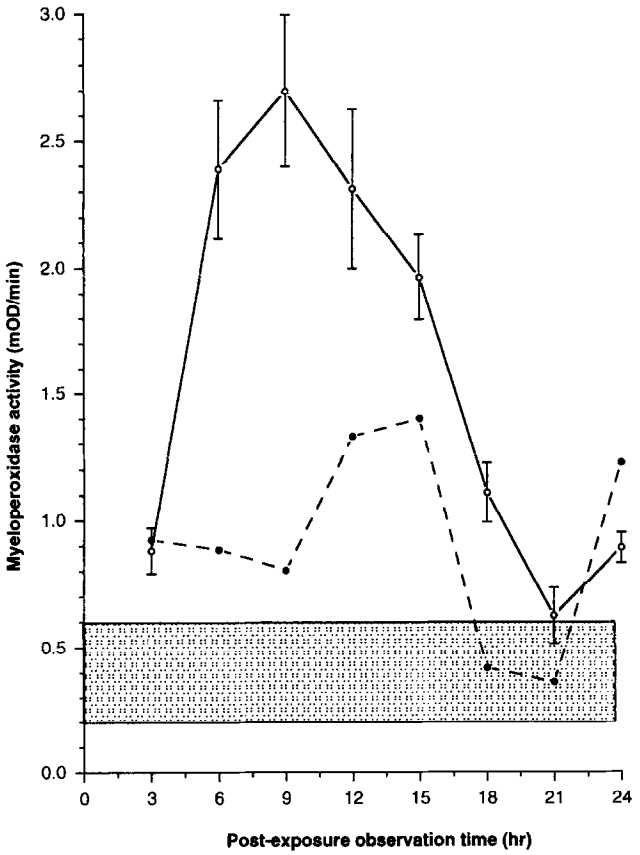

FIG. 2. Effect of BCES exposure on MPO levels in HGP skin: Comparison of sites exposed for 7 min to BCES vapor (open circles, solid line) with contralateral sham vapor cup sites (closed circle, broken line) from the same animals. Each time point from the exposed animals shows the mean ± SEM of 9–15 skin samples from a total of five different animals. Contralateral control points are shown as the mean of single skin samples from two different animals. The shaded box shows the 95% confidence interval for MPO activity of 15 skin samples obtained from a total of 8 naive control animals (no BCES exposure).

Using SDS–PAGE, there was little variation among subepidermal polypeptide extracts from different control animals (cf. Fig. 3A, lanes 3CON and 24CON). Furthermore, samples obtained 3, 6, or 9 hr after BCES exposure were identical to contralateral control sites. Increased silver staining at 180,000 M_r was observed in some subepidermal extracts obtained at 12–24 hr after BCES (Fig. 3A, 9H–21H).

Lectin Binding

Relative increases in the 180,000 M_r polypeptides were shown by the binding of con-A (Fig. 3B). Increased lectin binding could be resolved into two discrete polypeptides by prolonged electrophoresis of 6.5%T gels (Fig. 3B). Although comparisons were performed on the same sheets of PVDF or in tandem, a precise quantitation of lectin blots is precluded by a number of variables, including lectin and antibody dissociation constants, and length of time in substrate solution. Based upon detection of the known glycoproteins under conditions employed, the approximate sensitivity of the con-A method was established to be 50–100 ng protein if the PVDF was left for exactly 15 min in the substrate solution.

To assess whether increases in the extractable amounts of 180,000 M_r subepidermal polypeptides coincided with compensatory changes in the epidermis, we compared epidermal total protein extracts from the same animals. No consistent differences among control and BCES-treated sites were detected in the epidermal extracts by silver staining or by con-A lectin binding (Fig. 3C).

Antisera Binding

Three general conclusions were drawn from the application of several collagen antisera to HGP subepidermal extracts: (1) none differentiated between control and BCES-exposed sites; (2) several antisera to known extracellular matrix proteins from other rodents did not cross-react with HGP skin polypeptides (data not shown); and (3) the 180,000 M_r con-A-binding polypeptides did not react with antibodies to rodent collagens type I, III, or IV under conditions that do yield reactions with other HGP subepidermal polypeptides (Fig. 4).

Amino Acid Composition

Composition was determined for the 180,000 M_r polypeptides (Table 1). Five polypeptide bands were pooled for each determination, resulting in approximately 1.9 and 1.6 nmol total PTC-amino acids for the faster- and slower-migrating polypeptides, respectively. As reported previously, the background contamination of PVDF alone was only a fraction of the values determined for polypeptides [30]. The highest background was from glycine at 100 ± 10 pmol; no other amino acid produced more than 50 pmol background.

Comparison to SwissProt using PROPSEARCH revealed no known proteins with distance scores < 2.0 (70% reliability level) [32].

DISCUSSION

Because of its potency as a chemical warfare blistering agent, BCES has been the subject of considerable study and speculation [reviewed in Ref. 5]. By the end of the Second World War, it was deduced from *in vitro* and *in vivo* experiments that the basal epithelial cell nucleus is an early and primary target of BCES [35–37]. Subsequent work with isolated cells showed that BCES chemically modifies DNA, resulting in strand breaks and interstrand cross-links [reviewed in Ref. 38], as well as disrupting core histone-DNA interactions responsible for nucleosome structure [39]. There is no proof, however, that the nuclear effects of BCES lead to the tight, fluid-filled blisters in skin (or corneal detachment). Indeed, considerable evidence suggests that epidermal cell detachment may involve a mechanism(s) that is independent of mitotic inhibition, DNA

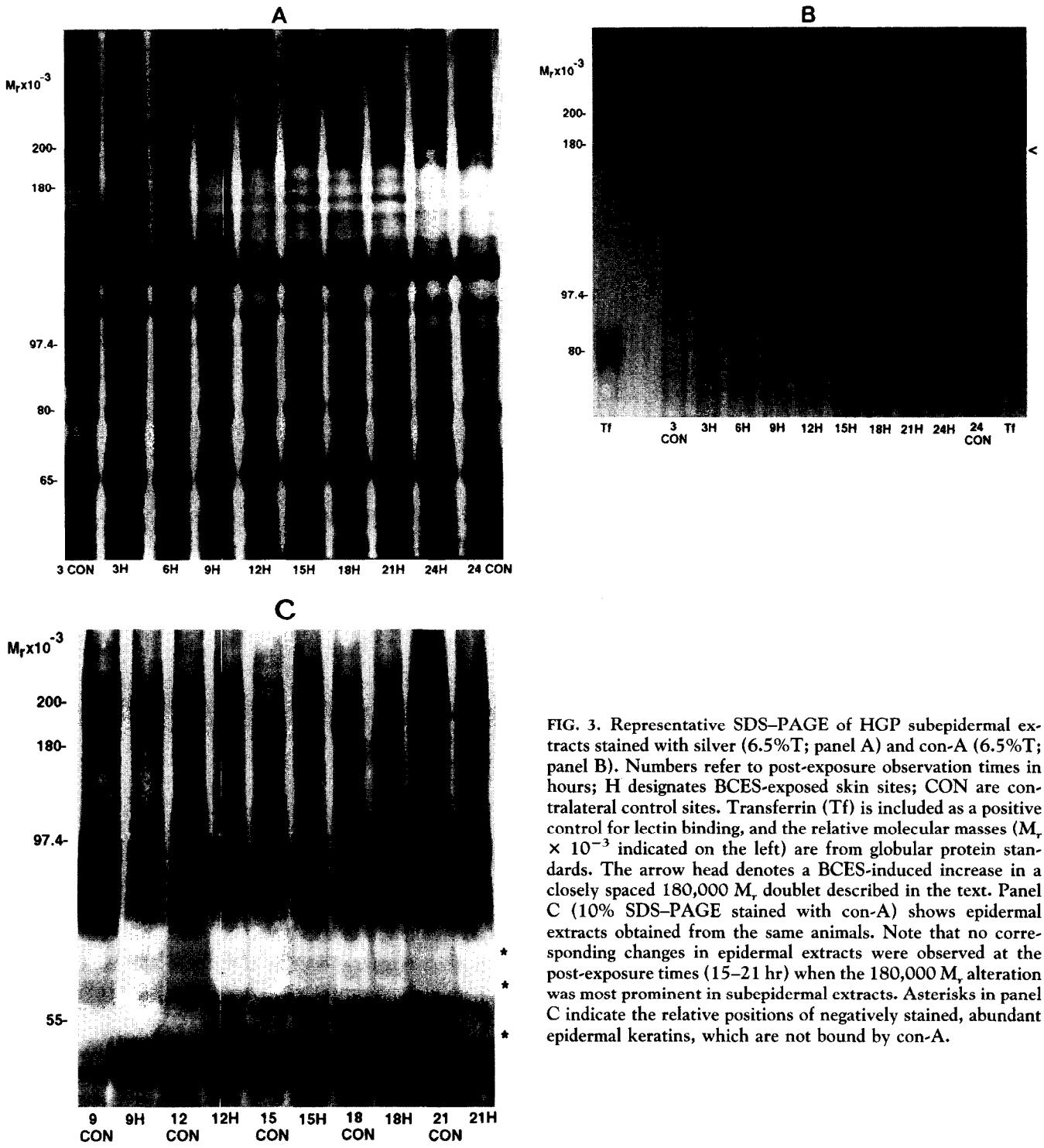

FIG. 3. Representative SDS–PAGE of HGP subepidermal extracts stained with silver (6.5%T; panel A) and con-A (6.5%T; panel B). Numbers refer to post-exposure observation times in hours; H designates BCES-exposed skin sites; CON are contralateral control sites. Transferrin (Tf) is included as a positive control for lectin binding, and the relative molecular masses ($M_r \times 10^{-3}$ indicated on the left) are from globular protein standards. The arrow head denotes a BCES-induced increase in a closely spaced 180,000 M_r doublet described in the text. Panel C (10% SDS–PAGE stained with con-A) shows epidermal extracts obtained from the same animals. Note that no corresponding changes in epidermal extracts were observed at the post-exposure times (15–21 hr) when the 180,000 M_r alteration was most prominent in subepidermal extracts. Asterisks in panel C indicate the relative positions of negatively stained, abundant epidermal keratins, which are not bound by con-A.

fragmentation, or the loss of basal cell viability caused by BCES [reviewed in Ref. 4]. We expect, therefore, that a complete description of BCES toxicity ultimately must include both DNA damage and specific molecules from subepidermal or inflammatory cells.

Latent and protracted tissue edema has been observed clinically in human skin exposed to saturated BCES vapor [reviewed in Ref. 5]. In our study, the HGP also showed a latent edema response with increased wet weight values at 50% of maximum by 9–12 hr after exposure and peak responses at 15–18 hr. Mice and rats, in contrast, generally show a more immediate edema response to BCES; for example, rat skin wet weight has been reported to reach 50% of maximum at 4–6 hr with peak values by 12 hr after a 5-mg BCES challenge [40].

Qualitative histopathology of tissues exposed to BCES consistently has shown an inflammatory cell infiltration of the papillary and reticular dermis [1, 6, 41], as well as of the

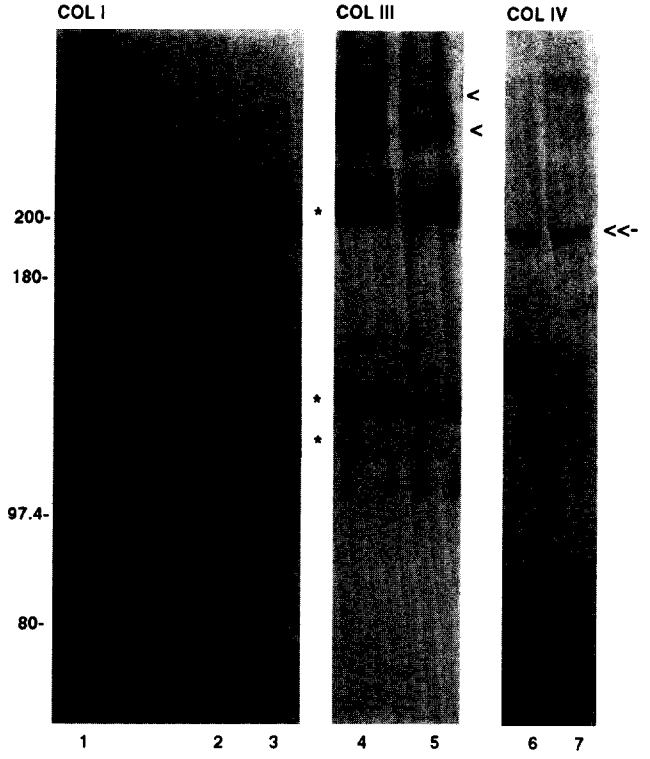

FIG. 4. Representative western blots of HGP subepidermal extracts stained with antisera to known rodent collagens. Extracts shown are from BCES-exposed sites (lanes 3, 5, and 7) and contralateral control sites (lanes 2, 4, and 6) at a 21-hr observation time. Each panel was stained with a different antiserum to either mouse collagen type I (COL I), rat skin collagen type III (COL III) or mouse tumor collagen type IV (COL IV). To facilitate comparison with Fig. 3B, relative molecular masses indicated on the left are from globular protein standards. Asterisks indicate the putative monomer and dimer of HGP skin collagen type I. As an internal standard, 50 μg of purified mouse collagen type I (Chemicon Int.) is shown in lane 1. Note that monomers of HGP collagen I and III appear to co-migrate but that additional high molecular weight polypeptides are bound by antiserum to collagen type III (single arrowheads). Collagen type IV antiserum bound a single 195,000 M_r polypeptide (double arrowhead), and no difference in relative molecular mass or immunoreactivity was observed for this polypeptide between exposed and contralateral control sites.

conjunctiva and cornea [11]. To quantify neutrophil infiltration in the HGP skin, we measured MPO activity directly and found it to be an early and reliable concomitant of vesicant-induced skin injury in the HGP.

At least two 180,000 M_r subepidermal polypeptides consistently increase in skin extracts after exposure to BCES. It is not known whether the observed alterations reflect a direct or indirect action of the vesicant, but the delayed time course of appearance coincides closely with the peak inflammatory response. High contents of glycine, proline, and hydroxyproline support the conclusion that both molecules are collagens (approximately 80–90% triple helical content). Presently, the 180,000 M_r collagens can be distinguished from known collagen types I, III, and IV because they are not bound by antisera under conditions that do yield specific reactions with polypeptides in the

TABLE 1. Amino acid determination of the 180,000 M_r polypeptides*†

Amino acid	Faster migrating polypeptide	Slower migrating polypeptide (residues/1000)
Asx	47	48
Glx	50	61
Hyp	74	95
Ser	59	50
Gly	253	302
His	10	11
Arg	48	47
Thr	40	25
Ala	89	98
Pro	116	117
Tyr	23	9
Val	45	34
Met	6	6
Ile	27	20
Leu	54	40
Hyl	5	9
Phe	25	trace
Lys	29	28
Cys‡		
Trp‡		

* Values are averages of three separate determinations.

† Raw data were adjusted by subtraction of average values obtained for each amino acid from adjacent "blank" PVDF [30].

‡ Not determined.

HGP subepidermal extracts (Fig. 4). Furthermore, the 180,000 M_r polypeptides bound avidly to con-A, whereas the major monomers of collagen types I and III did not (cf. Figs. 3B and 4). Because no single solubilization protocol can extract all cutaneous protein, our findings do not rule out the possibility that other extracellular matrix proteins also are altered by BCES exposure [cf. Ref. 42].

Peak amounts of the 180,000 M_r polypeptides occurred at 15–18 hr after BCES exposure. Although this alteration was detected with silver staining (Fig. 3A), it was more clearly shown by con-A binding to the subepidermal extracts (Fig. 3B). Based upon the lack of concurrent changes in the epidermal proteins bound by con-A (Fig. 3C), we do not believe the observed alteration is due to time-dependent changes in the overall protein extraction method. Furthermore, beyond 18 hr post-exposure, the relative amount of con-A bound at 180,000 M_r in subepidermal extracts consistently decreased (cf. Fig. 3B, lanes 15H and 24H). Interestingly, this response mirrored that observed for the inflammatory markers; tissue wet weight and MPO levels also consistently decreased between 15 and 24 hr after BCES exposure.

With regard to the source of the 180,000 M_r polypeptides, our experiments do not distinguish between new synthesis and degradation of subepidermal collagens, for example, by neutrophil collagenase(s). We note, however, that collagen type IV is a known target of neutrophil proteases [43], but it was not detectably degraded in HGP skin at 15–18 hr after BCES exposure (Fig. 4). This is

consistent with previous observations that the BCES-induced tissue cleavage plane occurs above the level of collagen type IV immunohistochemical staining in human skin explants [22] and in HGP skin [21].

Future studies of cutaneous neutrophil activation following BCES exposure should be undertaken for several reasons: (1) our results show that neutrophil infiltration consistently follows BCES exposure; (2) neutrophils oxidatively autoactivate latent collagenase through generation of hypochlorous acid ($H_2O_2 + Cl^- + H^+ \rightarrow HOCL + H_2O$; [44]); and (3) activated neutrophils generate long-lived *N*-chloramine compounds that inactivate leukocyte elastase inhibitor, α_1-PI, by oxidation of a critical methionine residue [45]. Along these lines, measurement of the α_1-PI family of inhibitors seems logical because BCES could exacerbate the effects of *N*-chloramine since the vesicant preferentially alkylates the sulfur-containing amino acids methionine and cysteine [46].

Measurements of cutaneous MPO activity as an early biological marker of BCES exposure should prove useful for routine screening of putative anti-inflammatory compounds. Utility of the altered collagens as biological markers, however, will depend upon isolation and identification.

Some forms of an inherited blistering disease, bullous pemphigoid, involve a well characterized 180,000 M_r hybrid collagen (called BP-180 or collagen type XVII) that is associated with hemidesmosome adhesion complexes and binds avidly to con-A lectin [47, 48]. Although both contain collagenous domains, the predicted amino acid composition of human BP-180 differs from that of the HGP hybrid collagens described in this report [48]. Compositional data, however, are insufficient to rule out common identity because of the possibility of species-specific isoforms or proteolytic fragmentation. The relationship, if any, between BP-180 and the HGP polypeptides altered by BCES exposure merits closer examination.

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References

1. Warthin AS and Weller CV, Histological studies of sulfur mustard exposure. *The Medical Aspects of Mustard Gas Poisoning*, pp. 35–80. C.V. Mosby Co., St. Louis, MO, 1919.
2. Buscher H, Review of sulfur mustard pathology. *Grun- und gelbkreuz*, pp. 1–50. Himmelheber & Co., Hamburg, Germany, 1921.
3. Cullumbine H, Medical aspects of mustard gas poisoning. *Nature* **159:** 151–153, 1947.
4. Friedenwald JS, Summary and some possible interpretations [of preceding series of studies on sulfur mustard]. *Bull Johns Hopkins Hosp* **82:** 326–352, 1948.
5. Papirmeister B, Feister AJ, Robinson SI and Ford RD, Histopathology of sulfur mustard exposure. *Medical Defense Against Mustard Gas*, pp. 48–74 and 174–191. CRC Press, Boca Raton, FL, 1991.
6. Henriques FC Jr, Moritz AR, Breyfogle HS and Patterson LA, The mechanism of cutaneous injury by mustard gas: An experimental study using mustard prepared with radioactive sulfur. *Progress Report for Contract NDCrc169 of National Defense Research Council*, DTIC No. ATI 30343, 1943.
7. Duke-Elder S, Vesicants. *Text-Book of Ophthalmology*, Vol. VI, pp. 6699–6713. C.V. Mosby Co., St. Louis, MO, 1954.
8. Willems JL, Clinical management of mustard gas casualties. *Ann Med Milit* **3:** 1–61, 1989.
9. Momeni AZ, Enshaeih S, Meghdadi M and Amindjavaheri M, Skin manifestations of mustard gas: A clinical study of 535 patients. *Arch Dermatol* **128:** 775–780, 1992.
10. Flury F and Wieland H, The pharmacologic action of dichlorethyl sulfide. *Z Exp Med* **13:** 367–483, 1921.
11. Maumenee AE and Scholz RO, The histopathology of the ocular lesions produced by the sulfur and nitrogen mustards. *Bull Johns Hopkins Hosp* **82:** 121–146, 1948.
12. McAdams AJ, A study of mustard vesication. *J Invest Dermatol* **26:** 317–326, 1956.
13. Smith KJ, Hurst CG, Moeller RB, Skelton HG and Sidell FR, Sulfur mustard: Its continuing threat as a chemical warfare agent, the cutaneous lesions induced, progress in understanding its mechanism of action, its long-term health effects, and new developments for protection and therapy. *J Am Acad Dermatol* **32:** 767–776, 1995.
14. Woodward D, Nieves A, Williams L, Spada C, Hawley S and Duenes J, A new hairless strain of guinea pig: Characterization of the cutaneous morphology and pharmacology. *Models Dermatol* **4:** 71–78, 1989.
15. Marlow DD, Mershon MM, Mitcheltree LW, Jaax GP and Petrali JP, Sulfur mustard induced skin injury in hairless guinea pigs. *J Toxicol Cut Ocular Toxicol* **9:** 179–192, 1989.
16. Mershon MM, Wade JV, Mitcheltree LW, Petrali J and Braue EH, Hairless guinea pig bioassay model for vesicant vapor exposure. *Fundam Appl Toxicol* **15:** 622–630, 1990.
17. Braue EH Jr, Koplovitz I, Mitcheltree LW, Clayson ET, Litchfield MR and Bangledorf CR, Characterization of the sulfur mustard vapor induced cutaneous lesions on hairless guinea pigs. *Toxicol Methods* **2:** 242–254, 1992.
18. Yourick JJ, Dawson JS and Mitcheltree LW, Sulfur mustard-induced microvesication in hairless guinea pigs: Effect of short-term niacinamide administration. *Toxicol Appl Pharmacol* **117:** 104–109, 1992.
19. Petrali JP, Oglesby SB and Mills KR, Ultrastructural correlates of sulfur mustard toxicity. *J Toxicol Cut Ocular Toxicol* **9:** 193–214, 1990.
20. Petrali JP, Oglesby SB, Hamilton TA and Mills KR, Comparative morphology of sulfur mustard effects in the hairless guinea pig and a human skin equivalent. *J Submicrosc Cytol Pathol* **25:** 113–118, 1993.
21. Petrali JP, Oglesby SB, Hamilton TA and Mills KR, Ultrastructural pathology and immunohistochemistry of mustard gas lesion. In: *Proceedings of the 50th Annual Meeting of the Electron Microscopy Society of America*, p. 826. San Francisco Press, San Francisco, CA, 1992.
22. Mol ME, de Vries R and Kluviers AW, Effects of nicotinamide on biochemical changes and microblistering induced by sulfur mustard in human skin organ cultures. *Toxicol Appl Pharmacol* **107:** 439–449, 1991.
23. Scaletta L, Occhino J, MacCallum D and Lillie J, Isolation and immunologic identification of basement membrane zone antigens from human skin. *Lab Invest* **39:** 1–9, 1978.
24. Woodley DT, Sauder D, Talley MJ, Silver M, Grotendorst G and Qwarnstrom E, Localization of basement membrane components after dermal-epidermal junction separation. *J Invest Dermatol* **81:** 149–153, 1983.
25. Gammon WR, Briggaman RA, Inman AO III, Queen LL and Wheeler CE, Differentiating anti-lamina lucida and anti-sublamina densa anti-BM antibodies by indirect immunoflu-

orescence on 1.0 M sodium chloride separated skin. *J Invest Dermatol* **82:** 139–144, 1984.
26. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
27. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
28. Mikol DD, Wrabetz L, Marton LS and Stefansson K, Developmental changes in the molecular weights of polypeptides in the human CNS that carry the HNK-1 epitope and bind *Phaseolus vulgaris* lectins. *J Neurochem* **50:** 1924–1928, 1988.
29. Millard CB, Tripathi BJ and Tripathi RC, Detection of glycated polypeptides in human aqueous humor by lectin binding analysis. *Exp Eye Res* **49:** 271–279, 1989.
30. Tous GI, Fausnaugh JL, Akinyosoye O, Lackland H, Winter-Cash P, Vitorica FJ and Stein S, Amino acid analysis of polyvinylidene difluoride membranes. *Anal Biochem* **179:** 50–55, 1989.
31. Heinrikson RL and Meredith SC, Amino acid analysis by reverse phase high performance liquid chromatography: Precolumn derivatization with phenylisothiocyanate. *Anal Biochem* **136:** 65–74, 1984.
32. Hobohm U, Hothaeve T and Sander C, Amino acid analysis and protein database composition search as a fast and inexpensive method. *Anal Biochem* **222:** 202–209, 1994.
33. Bradley PP, Priebat DA, Christensen RD and Rothstein G, Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78:** 206–209, 1982.
34. Box GEP, Hunter WG and Hunter JS, Significance tests and confidence intervals for means, variances, proportions and frequencies. In: *Statistics for Experimenters* (Eds. Box GEP, Hunter WG and Hunter JS), pp. 107–151. John Wiley, New York, 1978.
35. Gilman A and Philips FS, The biological actions and therapeutic applications of the B-chloroethyl amines and sulfides. *Science* **103:** 409–415, 1946.
36. Peters RA, Biochemical research at Oxford upon mustard gas. *Nature* **159:** 149–151, 1947.
37. Herriott RM and Price WH, The formation of bacterial viruses in bacteria rendered non-viable by mustard gas. *J Gen Physiol* **32:** 63–68, 1948.
38. Roberts JJ, Brent TP and Crathorn AR, The mechanism of the cytotoxic action of alkylating agents on mammalian cells. In: *The Interaction of Drugs and Subcellular Components in Animal Cells* (Ed. Cambell JN), pp. 5–27. J. & A. Churchill, London, 1968.
39. Millard CB, Meier HL and Broomfield CA, Exposure of human lymphocytes to bis-(2-chloroethyl)sulfide (sulfur mustard) solubilizes core histones. *Biochim Biophys Acta* **1224:** 389–394, 1994.
40. Young L, Observations on the effects of mustard gas on the rat. *Can J Res* **25:** 141–157, 1947.
41. Ginzler AM and Davis MIJ, The pathology of mustard burns of human skin. *Progress Report No. 3 to National Defense Research Council*, DTIC, 1943.
42. Zhang Z, Peters BP and Monteiro-Riviere NA, Assessment of sulfur mustard interaction with basement membrane components. *Cell Biol Toxicol* **11:** 89–101, 1995.
43. Murphy G, Ward R, Hembry RM, Reynolds JJ, Kuhn K and Tryggvason K, Characterization of gelatinase from pig polymorphonuclear leucocytes: A metallo proteinase resembling tumour type IV collagenase. *Biochem J* **258:** 463–472, 1989.
44. Weiss SJ, Peppin G, Ortiz X, Ragsdale C and Test ST, Oxidative autoactivation of latent collagenase by human neutrophils. *Science* **227:** 746–748, 1985.
45. Weiss SJ, Lampert MB and Test ST, Long-lived oxidants generated by human neutrophils: Characterization and bioactivity. *Science* **222:** 625–628, 1983.
46. Moore S, Stein WH and Fruton JS, Chemical reactions of mustard gas and related compounds: II. The reaction of mustard gas with carboxyl groups and with the amino groups of amino acids and peptides. *J Org Chem* **11:** 675–680, 1946.
47. Guidice GJ, Emery DJ and Diaz LA, Cloning and primary structural analysis of the bullous pemphigoid autoantigen, BP180. *J Invest Dermatol* **99:** 243–250, 1992.
48. Hopkinson SB, Baker SE and Jones JCR, Molecular genetic studies of a human epidermal autoantigen (the 180-kD bullous pemphigoid antigen/BP180): Identification of functionally important sequences within the BP180 molecule and evidence for an interaction between BP180 and α6 integrin. *J Cell Biol* **130:** 117–125, 1995.