Airway Obstruction Due to Bronchial Vascular Injury after Sulfur Mustard Analog Inhalation

Livia A. Veress^{1,2}, Heidi C. O'Neill^{2,3}, Tara B. Hendry-Hofer², Joan E. Loader², Raymond C. Rancourt², and Carl W. White¹⁻³

¹Department of Pediatrics, University of Colorado Health Sciences Center, ²Department of Pediatrics, National Jewish Health, and ³Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Colorado

Rationale: Sulfur mustard (SM) is a frequently used chemical warfare agent, even in modern history. SM inhalation causes significant respiratory tract injury, with early complications due to airway obstructive bronchial casts, akin to those seen after smoke inhalation and in single-ventricle physiology. This process with SM is poorly understood because animal models are unavailable.

Objectives: To develop a rat inhalation model for airway obstruction with the SM analog 2-chloroethyl ethyl sulfide (CEES), and to investigate the pathogenesis of bronchial cast formation.

Methods: Adult rats were exposed to 0, 5, or 7.5% CEES in ethanol via nose-only aerosol inhalation (15 min). Airway microdissection and confocal microscopy were used to assess cast formation (4 and 18 h after exposure). Bronchoalveolar lavage fluid (BALF) retrieval and intravascular dye injection were done to evaluate vascular permeability.

Measurements and Main Results: Bronchial casts, composed of abundant fibrin and lacking mucus, occluded dependent lobar bronchi within 18 hours of CEES exposure. BALF contained elevated concentrations of IgM, protein, and fibrin. Accumulation of fibrin-rich fluid in peribronchovascular regions (4 h) preceded cast formation. Monastral blue dye leakage identified bronchial vessels as the site of leakage.

Conclusions: After CEES inhalation, increased permeability from damaged bronchial vessels underlying damaged airway epithelium leads to the appearance of plasma proteins in both peribronchovascular regions and BALF. The subsequent formation of fibrin-rich casts within the airways then leads to airways obstruction, causing significant morbidity and mortality acutely after exposure.

Keywords: fibrin; pseudomembrane; plastic bronchitis; vascular permeability; microdissection

Sulfur mustard, bis(2-chloroethyl) sulfide (SM), is a chemical agent used in modern warfare, most recently by Iraq in the 1983–1988 Iran–Iraq war (1–6). It is a vesicant, affecting mainly the skin, eyes, and respiratory system shortly after direct contact (3, 7, 8). In high doses, SM exposure can lead to multiorgan involvement (3, 4, 8, 9) and can result in death (3, 6, 10–12). There are 40,000–50,000 surviving victims of SM inhalation in Iran and Iraq alone, many with permanent pulmonary disabilities such as bronchiolitis obliterans (13, 14).

Respiratory effects of SM exposure have long been investigated, focusing mainly on chronic respiratory effects in survivors

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Among the effects of sulfur mustard (SM) on the respiratory tract, airway obstructive cast development after acute exposure remains poorly understood.

What This Study Adds to the Field

We demonstrate a rat model for SM surrogate agent-induced bronchial cast formation, and show that bronchial vascular injury occurring early after exposure leads to the development of airway-obstructive casts.

(1, 2, 13–16). Regarding the acute effects of SM on the human respiratory tract, scarce data are available other than a few case reports (2–4, 6, 8, 10, 11, 17). Injury appears concentration dependent (5, 7), with low-level SM exposure affecting mainly the upper respiratory tract. This can result in nasal mucosal injury, rhinorrhea, loss of smell and taste, pharyngeal mucosal injury, and laryngitis (2, 3, 7, 10). Moderate SM exposure results in various degrees of tracheobronchial mucosal injury, leading to a painful and forceful cough (2, 3, 7, 15). By contrast, high-level SM exposure can often lead to more severely disabling respiratory lesions that may cause death (6, 8, 10, 12). These effects include severe airway edema and ulceration, tracheobronchial mucosal sloughing, and airway-occlusive pseudomembranes (2, 4–7, 10–12, 15, 17–19).

Although high-dose SM can cause fatality from multiorgan failure alone (2, 6), numerous case reports have also documented sudden deaths occurring from acute airway obstruction due to pseudomembrane formation (6, 7, 10, 12, 20). The etiology of this airway occlusion remains poorly understood, as human exposure in more recent years has been fortunately infrequent, and no animal model has yet been developed to specifically mimic this phenomenon. Therefore, no therapies have been evaluated in this process, and none exist to prevent or alleviate this potentially fatal event. As SM continues to be a potential agent in bioterrorism (5, 21), an improved understanding of this disorder could allow development of effective therapeutic interventions.

2-Chloroethyl ethyl sulfide (CEES) is a surrogate agent used to mimic SM injury in laboratories (22, 23). It is less toxic than SM because of the absence of one of two terminal chloride groups (that cross-link DNA and proteins), and because of its considerably shorter half-life in aqueous solution. Although safer to handle than SM, CEES possesses many of the same damaging properties as an alkylating agent (22, 23) as does SM, making it suitable for use in experiments to mimic SM-induced respiratory tract injury without the need for a specialized containment facility. In this article, we provide an animal model for potentially fatal airway obstruction seen acutely after SM

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Correspondence and requests for reprints should be addressed to Carl W. White, M.D., Department of Pediatrics, Pulmonary Division, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206. E-mail: whitec@njhealth.org

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exposure using the SM surrogate agent, CEES. In addition, we examine the composition of these pseudomembranous casts, and demonstrate the significant role that early injury to the bronchial circulation plays in their formation.

METHODS

An expanded methods description can be found in the online supplement.

Chemicals

2-Chloroethyl ethyl sulfide (CEES, 8.41 M) was obtained from TCI America (Portland, OR). All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise indicated.

Animal Care

The Institutional Animal Care and Use Committee (IACUC) of National Jewish Medical and Research Center (Denver, CO) approved this study. Adult male (275 to 350-g) Sprague-Dawley rats (Harlan Co., Indianapolis, IN) were used.

Inhalation Exposure to CEES

Rats were anesthetized with a cocktail of ketamine (75 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.5 mg/kg) and placed in polycarbonate tubes with sealing plungers. Tubes containing animals were mounted in a nose-only inhalation system (CH Technologies, Westwood, NJ), and were administered compressed air with the aerosolized compound (ethanol, or 5 or 7.5% CEES in ethanol) for 15 minutes. Aerosolization was conducted via a Razel syringe pump (Razel Scientific, St. Albans, VT) connected to a BioAerosol nebulizing generator (BANG; CH Technologies). After 15 minutes of exposure, rats were removed from the polycarbonate tubes and were observed in their cages until they had fully recovered from anesthesia.

Lung Fixation

Animals were killed 4, 18, or 72 hours after exposure as per experimental design. If the rats became moribund, as demonstrated by weight loss greater than 25% body weight, inability to eat or drink, and so on, they were killed before the planned study termination as per IACUC protocol. Rats within experimental groups were terminally anesthetized with pentobarbital (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA), the tracheas were cannulated, and lungs were fixed at 20 cm H₂O with either Karnovsky's fixative or 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 and 30 minutes, respectively. Whole lungs were then removed by gross dissection.

Airway Microdissection

The protocol for microdissection as described by Postlethwait and colleagues (24, 25) was followed. Microdissection was performed on a Petri dish. Beginning at the main lobar bronchus (generation 3), the axial pathway was exposed by cutting the airway lumen at the 3 and 9 o'clock positions. Main daughter branches, or side branches, were also exposed during the microdissection. A map of airway generations was drawn during microdissection (Figure 1).

Confocal Microscopy Using Ethidium Homodimer-1 and YO-PRO-1

Distribution of airway injury was assessed by a three-dimensional imaging technique modified after Postlethwait and colleagues (24, 25). Main modifications to the previously described protocol included the use of 6 μ M ethidium homodimer-1 (EthD-1, 20 ml/kg; Molecular Probes Inc., Eugene, OR) in phenol red-free RPMI medium used for vital staining of airways and their contents. In addition, we also used 2 μ M YO-PRO-1 (Molecular Probes Inc.) solution in PBS for staining of right middle lobe airways after microdissection but before imaging. A two-photon LSM 510 confocal microscope (Zeiss, Thornwood, NY) was used to obtain images and z-stacks. For bronchial cast imaging, casts were removed via microdissection from their airway locations after EthD-1 labeling *in situ*, and then incubated with YO-PRO-1 solution and imaged with the confocal microscope.

Differential Cell Counts in Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) was pooled and centrifuged and the pellet was washed in 2 ml of PBS; the pellet was then resuspended in 2 ml of PBS and centrifuged in a Cytospin (Shandon Scientific) followed by staining with modified Wright-Giemsa stain (Protocol Hema 3; Fisher Scientific, Fair Lawn, NJ). Cell counts were then obtained via hemacytometer, counting 200 cells minimum in three random fields.

IgM and Total Protein Measurement in Bronchoalveolar Lavage Fluid

Two lavages, each with 5 ml of PBS, were instilled into the lungs via the tracheal cannula and subsequently withdrawn and then pooled. IgM concentrations were quantitatively measured in the BALF according to a standard ELISA protocol (Bethyl Laboratories, Inc., Montgomery, TX). Total protein was measured in BALF, using the bicinchoninic acid (BCA) protein assay (Pierce, Rockville, IL).

β -Fibrin Detection by Western Blot

β -Fibrin was detected in BALF by Western blot with polyclonal rabbit anti-human fibrinogen (DAKO, Glostrup, Denmark) or mouse monoclonal β -actin (Sigma), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) or horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma), respectively. Rat fibrinogen (Sigma-Aldrich Chemical Co.) was used to generate fibrin standards. Results were normalized to the protein level within each sample.

Fibrinogen/ β -Fibrin and Acetylated Tubulin Immunohistochemistry

Avidin-biotin complex peroxidase methods were used on tissue sections for staining based on a VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA). The primary antibody used for fibrin(ogen) immunohistochemistry (IHC) was polyclonal rabbit anti-human fibrinogen (DAKO), and for acetylated tubulin IHC it was monoclonal mouse acetylated α -tubulin (Abcam, Cambridge, MA), for 60-minute incubations each. The secondary antibody used was biotinylated horse anti-mouse IgG. Counterstaining was performed with hematoxylin.

Myeloperoxidase Activity Assay

Snap-frozen lung tissue was homogenized and centrifuged, the supernatant was withdrawn, and the steps were repeated until clear. The 1-ml reaction cuvette (with PBS, H₂O₂, and tetramethylbenzidine) was monitored for 3 minutes at 652 nm, using a DU-64 spectrophotometer (Beckman Coulter, Fullerton, CA). Calculated milliunits of activity were normalized to milligrams of protein, using the BCA protein assay (Thermo Scientific).

Tissue Preparation for Histology

Paraffin-embedded tissues were sectioned at a thickness of 5 μ m and then stained with hematoxylin and eosin. Additional slides were also stained with combined alcian blue and periodic acid-Schiff stain for the localization of acidic and neutral mucins, respectively, and counterstained with hematoxylin. In addition, Movat's pentachrome staining was performed on 72-hour lung sections for assessment of collagen deposition.

Evans Blue Dye Extravasation

Vascular permeability changes were assessed by monitoring the extravasation of Evans blue dye (Sigma-Aldrich Chemical Co.), using a method modified after Evans and colleagues (26, 27). Briefly, 45 minutes before they were killed and their lungs collected, the animals were injected via the tail vein with Evans blue dye (30 mg/kg). After fixation, lungs were microdissected and airway images were obtained with an SMX 1500 camera (Nikon Instruments, Inc., Melville, NY).

Monastral Blue B Labeling of Permeable Vessels

Thirty minutes before they were killed, the animals were injected via the tail vein with monastral blue B suspension (30 mg/kg; Sigma-

Figure 1. Gross specimen of a microdissected right middle lobe after exposure (18 h) to 2-chloroethyl ethyl sulfide (7.5%). Bronchial cast material (arrow) is present within the exposed central airway to axial generation 15. Daughter branches show extension of cast formation for an additional four to eight generations.

Aldrich Chemical Co.), a compound used to label sites of vascular leak, at a concentration of 1 mg/ml (28). Animals were killed 4 hours after exposure and lungs were harvested, fixed, microdissected, and imaged as described.

Statistical Analysis

For statistical analysis, Prism 5.01 software (GraphPad, La Jolla, CA) was used. Results are presented as means $\pm$ SEM in the text and figures. Groups were subjected to one-way analysis of variance, and when significance was found, Tukey's *post hoc* analysis was applied. *P* values less than 0.05 were considered significant.

RESULTS

Assessment of Airway Cast Formation

Eighteen hours after CEES inhalation, we found bronchial casts within all lobes, especially within dependent lung regions such as the right lower lobe, the lower portion of the left lobe, and the accessory lobe (Figure 2B). Cast formation with 5% CEES was inconsistent in both location and degree of obstruction. In contrast to 5% CEES, 7.5% CEES inhalation produced reliable cast formation and more severe injury, with larger bronchial casts that often caused complete airway obstruction of some lobes. Again, this was particularly evident within the dependent lobes. Complete occlusion of all lobes was incompatible with survival and was noted during necropsy of several nonsurviving animals. The mortality rate with 7.5% CEES was 25% at 18 hours and 67% at 72 hours, whereas 5% CEES caused no mortality at all time points examined (Table 1). With ethanol exposure alone, no cast formation was observed in any airways (Figure 2A). Detailed mapping of bronchial casts within the airways revealed that such casts extended from the tracheal bifurcation to, at most, airway generation 15 of the axial pathway (Figure 1). Major daughter generations also contained extensions of the same casts for up to an additional four distal generations.

Composition of Airway Casts

Because bronchial cast composition is likely related to underlying mechanism(s) resulting in their formation, we next sought to classify the casts formed after CEES exposure. After

Figure 2. Gross specimen of cross-sectioned accessory lobe main bronchi after aerosol exposure (18 h) to (A) diluent (ethanol) or (B) 2-chloroethyl ethyl sulfide (CEES, 5%). Complete airway occlusion from cast material is noted (arrow) with CEES.

rats were exposed to 5% CEES for 18 hours, the lungs were fixed and microdissection was performed on the right middle lobe. Bronchial casts were then carefully removed in their entirety, and processed for immunohistochemistry, histology, or confocal microscopy. Immunohistochemical examination revealed fibrin(ogen) in great abundance as a component of these casts (Figures 3A and 3B). Periodic acid-Schiff/alcan blue staining of cast sections did not demonstrate mucus staining at 18 hours (Figure 3C), indicating that casts formed after CEES exposure were not mucin-based. Hematoxylin and eosin staining of airway cast sections demonstrated scattered inflammatory cells dispersed throughout entire casts. The inflammatory cells appeared to be concentrated heavily along the edges of the casts, particularly by 72 hours after exposure (Figure 3E). Collagen deposition was noted by 72 hours, with the appearance of spindle cells suggestive of myofibroblasts or fibroblasts within the casts (Figure 3F). We also noted occasional clumps of ciliated epithelial cells by 18 hours (Figures 3C and 3D), deeply embedded within the periphery of the casts. The presence of ciliated epithelial cells was confirmed via immunohistochemical staining for acetylated tubulin present in cilia (*see* Figure E1 in the online supplement).

To assess whether the cells within these casts and their adjacent airways were still viable, we employed a confocal

TABLE 1. MORTALITY FROM 2-CHLOROETHYL ETHYL SULFIDE IN RATS

Time Post-CEES Inhalation (h)	Mortality Rate		
	Ethanol	CEES (5%)	CEES (7.5%)
4	0% (0/14)	0% (0/14)	0% (0/8)
18	0% (0/14)	0% (0/11)	25% (7/28)
72	0% (0/8)	0% (0/10)	67% (4/6)

Definition of abbreviation: CEES = 2-chloroethyl ethyl sulfide.

microscopy double-staining technique using YO-PRO-1 and EthD-1 nuclear dyes to indicate live (green) or dead (red) cells, respectively (24, 25). YO-PRO-1 nuclear dye was used to stain all cell nuclei as a “background” stain by study design, and EthD-1 nuclear dye was used to indicate dead or dying cells with compromised cell membrane integrity. We found that the majority of cells within the casts were YO-PRO-1 positive, or live cells, especially those cells located at the periphery of the casts (Figure E2). Dead cells, which stained positively with EthD-1, were only sporadically noted, and were seen mostly within the core of the cast, where cells identified by histology appeared to be mainly inflammatory in origin.

Plasma-derived Protein Quantitation in BALF

The presence of fibrin-rich casts within airways after CEES exposure implied leakage of the fibrin precursor protein, fibrinogen (340 kD as dimer), from the surrounding vasculature into the airway lumen, because fibrinogen is normally found only within blood plasma. Therefore, we next sought to quantify the amount of fibrin(ogen) within BALF after various inhaled CEES concentrations, using Western blotting and densitometry for quantitation. We also assessed the concentration of total protein present in BALF, as well as that of IgM, a high molecular weight immunoglobulin normally confined to the circulation (700 kD as pentamer). At both 4 and 18 hours, we found a significant dose-related increase in BALF protein, IgM, and most notably β -fibrin at both CEES concentrations tested. As compared with levels in ethanol-exposed rats, with 5% CEES exposure we observed a threefold increase at 4 hours and a sixfold increase at 18 hours in BALF total protein concentration (Figure 4A). After 7.5% CEES inhalation, protein in BALF was increased 4-fold at 4 hours and 10-fold at 18 hours over ethanol. BALF obtained from naive rats contained total protein at concentrations similar to those found after ethanol exposure, which were minimal. When IgM concentrations were measured in BALF, there was a 3-fold increase with 5% CEES at 4 hours, and a 19-fold increase at 18 hours as compared with ethanol (Figure 4B). The BALF IgM levels further increased with 7.5% CEES to 4-fold at 4 hours and 31-fold at 18 hours. IgM was not detected in BALF from naive rat lungs. The concentration of β -fibrin (normalized to protein content) also was significantly increased in BALF after CEES exposure (Figure 4C). Inhalation of 5% CEES resulted in a 4-fold increase at 4 hours and a 10-fold increase at 18 hours in BALF β -fibrin concentration compared with ethanol levels. After 7.5% CEES inhalation, this increase was 5-fold at 4 hours and 12-fold at 18 hours over ethanol levels. Again, β -fibrin concentrations in BALF from naive rats were comparable to those observed after diluent (ethanol) exposure.

Cell Differential Counts and Myeloperoxidase Activity

To assess the role of inflammation in cast formation, we analyzed differential cell counts of inflammatory cells in BALF from both 5 and 7.5% CEES-exposed rat lungs at 4 and 18

Figure 3. Photomicrographs of central airway casts removed from central airways after 5% 2-chloroethyl ethyl sulfide (CEES) aerosol exposure, at (A–D) 18 hours or (E and F) 72 hours, and then processed for immunohistochemistry (IHC) or stained with periodic acid–Schiff/alcian blue (PAS/AB) or hematoxylin and eosin (H&E). (A) IHC staining for fibrin within the cast. (B) IHC staining for nonspecific anti-rat IgG rather than antibodies directed against fibrinogen/fibrin (nonspecific controls). (C) PAS/AB staining of cast at 18 hours, showing no mucin staining. A ciliated epithelial cell clump incorporated within the cast is seen (arrow), as well as adherent inflammatory cells. (D) H&E staining of cast at 18 hours, with a notable incorporated epithelial cell clump (arrow). (E) H&E staining of cast at 72 hours, with a large increase in inflammatory cells noted. (F) H&E staining of cast at 72 hours, with the appearance of spindle cells suggestive of fibroblasts/myofibroblasts within the cast matrix (arrows). All pictures were taken at an original magnification of $\times 40$.

hours (Figure E3). Macrophage levels gradually declined over time and with higher CEES concentrations. The BALF absolute macrophage counts with 7.5% CEES showed a twofold (4 h) and a threefold (18 h) reduction over ethanol-exposed levels, whereas with 5% CEES no change in macrophages was observed at 4 hours, and only a modest decrease at 18 hours (1.3-fold). In contrast, the BALF percent and absolute polymorphonuclear leukocyte (PMN) count increased in a time-dependent fashion but without a significant CEES dose–response pattern (Figures 5A and E3). Only a minimal increase in PMNs was detected in BALF at the 4-hour time point with either 5 or 7.5% CEES. However, at 18 hours there was a significant 15-fold increase in percent BALF PMNs with both 5 and 7.5% CEES. Ethanol exposure caused no measurable increase in BALF PMNs, and showed comparable macrophage levels to those in naive rats (data not shown).

To assess whole lung inflammation, we next evaluated the levels of myeloperoxidase (MPO) in lung homogenates after CEES exposure. MPO is a peroxidase enzyme present predominantly in neutrophils, and thereby serving as a useful marker for the presence of these granulocytes. Relative to ethanol exposure, 5% CEES inhalation resulted in a 3-fold (4 h)

Figure 4. Airway protein components appearing 4 and 18 hours after 2-chloroethyl ethyl sulfide (CEES) exposure. (A) Effect of CEES on total protein concentration in bronchoalveolar lavage fluid (BALF). Analysis done by bicinchoninic acid binding assay. (B) Effect of CEES on IgM concentration in BALF. Analysis by ELISA. (C) Effect of CEES on β -fibrin in BALF. Analysis done via Western blotting, with densitometry used to obtain concentration values. Values represent means $\pm$ SEM ($n = 6$ per group, except in the β -fibrin study for the CEES group [$n = 3$]). *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$ for comparison of both CEES-exposed groups versus both naive and ethanol groups. EtOH = ethanol.

and a 19-fold (18 h) increase in MPO, whereas 7.5% CEES inhalation resulted in a 2-fold (4 h) and a 14-fold (18 h) increase. Levels from naive animals were comparable to those in rats exposed to ethanol (data not shown).

Assessment of Vascular Permeability with Evans Blue Dye

As localization of fibrin within the airway implies vascular injury, we next sought to examine vascular permeability after CEES inhalation by tracing the extravasation of Evans blue dye from permeable vessels. Evans blue dye binds to serum albumin (66 kD), and its leakage implies that blood vessels are permeable to proteins of this size or greater. Because casts were “well formed” by 18 hours, and plasma proteins were a major component of the casts, we assumed that vascular leakage must precede cast formation. Therefore, we assessed for increased

Figure 5. Bronchoalveolar lavage fluid polymorphonuclear leukocyte (PMN) and whole lung myeloperoxidase (MPO) levels after inhalation of 2-chloroethyl ethyl sulfide (CEES). (A) Effect of CEES (5 and 7.5%) on percent PMNs at both 4 and 18 hours after exposure. (B) Levels of MPO activity in homogenized whole lung after CEES (4 and 18 h; 5 and 7.5%) is shown normalized to protein levels. Values represent means $\pm$ SEM ($n = 6$ per group), with *** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$ for comparison of both CEES-exposed groups versus the ethanol (EtOH) group.

vascular permeability at 4 hours via the Evans blue dye extravasation method, before the appearance of any casts. Animals were injected via the tail vein with Evans blue dye (30 mg/kg) 45 minutes before necropsy and after exposure to CEES or ethanol. Microdissection of all lobes was then performed to localize dye leakage. After CEES exposure, we noted extravasation of Evans blue dye around the distal trachea and central bronchi (Figures E4C and E4D), but no dye was detected after ethanol-only exposure (Figures E4A and E4B). This effect was CEES concentration dependent, in that dye extravasation was greater in the 7.5% CEES group (data not shown). In addition to peribronchial and peritracheal staining, regions around the central pulmonary vessels (both arteries and veins) also demonstrated increased Evans blue staining after CEES inhalation compared with both ethanol-exposed and naive controls (Figures E5A and E5B). No parenchymal staining was noted at any concentration tested, indicating that increased permeability did not occur in the pulmonary microcirculation.

Histological Assessment of Vascular Leakage

As Evans blue dye is albumin bound, it is a nonspecific indicator of vascular permeability. Nevertheless, it was useful in localizing vascular injury to peribronchial regions after CEES exposure. Damage to the bronchial vasculature was further suggested by examination of histological sections of accessory lobes stained with hematoxylin and eosin, which demonstrated

Figure 6. Fibrin deposition detected by immunohistochemical staining in lung sections of accessory lobe main bronchi in (A) diluent (ethanol; 18 h), (B) 2-chloroethyl ethyl sulfide (CEES) (5%; 4 h), (C) CEES (5%; 18 h), and (D) CEES (5%; 72 h), as well as Movat's pentachrome staining obtained 72 hours after diluent (F) or CEES (E, 5%) exposure. Intense fibrin staining is illustrated in peribronchovascular space (arrowheads) after CEES, as well as around large pulmonary vessels (thick arrows) adjacent to the main bronchi (asterisks). (C) and (D) show airway cast (thin arrows) with strong fibrin staining (original magnification, $\times 10$). (E) Peribronchial and subepithelial (large arrows) deposition of both immature (blue) and mature (yellow) collagen, as well as denuded ciliated epithelium (arrowhead) and airway luminal cast (left side), neither of which is seen in lungs exposed to diluent alone (F).

significant edema formation within the peribronchovascular space, both 4 and 18 hours after 5% CEES exposure (Figures E6C and E6D, respectively). Although the eosinophilic staining

of this fluid appeared to be patchy in distribution, its presence probably indicates an elevated protein content of the fluid. In some areas, eosinophilic staining of such fluid accumulation was less intense, possibly because of postfixation and/or processing artifact. In eosinophilic staining regions, edema formation appeared to be particularly intense adjacent to the adventitial layer of large pulmonary vessels, with relatively less edema noted within the immediate peribronchial space. No edema was noted in any of these areas in lungs of either naive or ethanol-exposed animals (Figures E6A and E6B, respectively).

Because bronchial casts were found to be composed of abundant fibrin, we next used immunohistochemistry to more precisely localize fibrin(ogen) in the peribronchovascular regions. Accessory lobes of ethanol-exposed and 5% CEES-exposed lungs were assessed 4, 18, and 72 hours after exposure. By 4 hours, we detected increased fibrin(ogen) staining within the peribronchovascular space, which was particularly intense within the perivascular regions (Figure 6B). As expected, ethanol-exposed rat lungs did not demonstrate fibrin(ogen) staining outside blood vessels (Figure 6A). By 18 hours after CEES exposure, when casts were well formed, we noted fibrin(ogen) staining persisting within the peribronchovascular space (Figure 6C), similar to that seen after 4 hours, both as to location and intensity. By 72 hours, fibrin staining within peribronchovascular spaces diminished (Figure 6D), but strong fibrin(ogen) staining remained within airway casts. Within peribronchovascular spaces where fibrin(ogen) staining was evident earlier, increased collagen deposition was now detected by pentachrome staining. Both mature (yellow-staining) and immature (blue-staining) collagen was noted, particularly within the thickened subepithelial interstitium 72 hours after CEES exposure (Figure 6E). No increased collagen deposition was noted with ethanol exposure in any regions (Figure 6F).

Detection of Increased Vascular Permeability by Monastral Blue Pigment

Although we could localize both edema and fibrin(ogen) staining within the peribronchovascular space by histology and Evans blue dye labeling, we were unable to identify which specific vessel(s) had increased permeability after CEES exposure by these methods. Therefore, we employed monastral blue pigment as a tracer to label vessels with increased permeability (30 mg/kg, intravenous). Monastral blue pigment is unique in that it readily crosses the endothelium of abnormally perme-

Figure 7. Gross specimen of accessory lobe main stem bronchi after monastral blue pigment injection 4 and 18 hours after exposures to 2-chloroethyl ethyl sulfide (CEES). Control rat airway (diluent exposed) shown (A) in cross-section and (D) *en face* 4 hours after monastral blue injection. CEES-exposed (5%) rat airway shown in cross-section and *en face* 4 hours (B and E, respectively) and 18 hours (C and F, respectively) after pigment injection. Note the diffuse monastral blue labeling of bronchial vessels (yellow arrows) within the peribronchial space at both 4 and 18 hours after CEES exposure. A bronchial cast (black arrow) is present at 18 hours (C) within luminal air bubbles present.

able blood vessels, but it can then become trapped within the basal lamina of these vessels, thereby labeling these sites of extravasation. We assessed rat lungs for vascular injury 4 and 18 hours after CEES or ethanol exposure, 30 minutes after monastral blue pigment injection. With the aid of microdissection, we observed numerous monastral blue-labeled vessels immediately beneath the airway epithelium 4 hours after CEES exposure (Figures 7B and 7E), consistent with the anatomic location of the airway bronchial vascular plexus (29). This intensity of monastral blue labeling of bronchial vessels persisted at 18 hours (Figures 7C and 7F), indicating a sustained increase in permeability, and therefore injury, of bronchial vessels. Monastral blue-labeled vessels extended from the distal one-third of trachea (Figure E7B) with deposition distally to airway generation 12 of each lobar bronchus. This labeling was consistent with the location of the bronchial circulation. In the trachea, we observed increased monastral blue staining within the intercartilaginous mucosal regions, with less intense staining within the cartilage rings themselves at both 4 and 18 hours (Figures E8B and E8D, respectively). Monastral blue staining was concentration related with respect to CEES inhalation, with increased vascular staining intensity after 7.5% CEES exposure. After ethanol exposure alone, no monastral blue labeling was detected in any lung regions at either of these time points (Figures 7A and 7D; and *see* Figures E7A, E8A, and E8C). No pulmonary artery or pulmonary vein labeling was noted at any concentration of CEES tested. In addition, monastral blue labeling was never observed within the pulmonary parenchymal microcirculation, again indicating a lack of pulmonary microvascular injury often seen in many forms of inhalation insult associated with acute lung injury.

Confocal Microscopic Airway Analysis

With use of monastral blue pigment labeling, we were able to identify injured bronchial vessels with increased permeability in subepithelial regions. If the vessels in subepithelial regions were most affected after inhalation of this noxious agent, we hypothesized that the epithelial surface itself was also potentially altered. Therefore, we next sought to identify the distribution, type, and intensity of epithelial surface injury after CEES inhalation exposure by confocal microscopy. Microdissection was used to expose axial and daughter pathways of the right middle lobe of each rat lung within the naive, 5% CEES-exposed, and ethanol-exposed groups 18 hours after inhalation. We evaluated the distribution of EthD-1-positive cells on the costal surface of each right middle lobe. Again, double staining was performed with YO-PRO-1 and EthD-1 nuclear dyes to assess for cell death. YO-PRO-1 nuclear dye was used to stain all cell nuclei as a “background” stain by study design, and EthD-1 nuclear dye was used to indicate dead or dying cells with compromised cell membrane integrity. We found that, after 5% CEES exposure, casts were present within airway lumens by 18 hours. After careful removal of these casts, large collections of EthD-1-positive cells were noted at the site of cast attachment to the epithelial layer at generation 3 of the main lobar bronchus (Figure E9). Distal to generation 3, no EthD-1-positive cells were seen within either axial or daughter pathways (Figure 8C). More importantly, numerous gaps or voids were noted within the epithelial layer after CEES exposure, where a lack of YO-PRO-1 staining was evident. These defects were approximately 1–10 cell diameters in size. Although they were distributed throughout all daughter and axial generations, they appeared more prominently within the proximal axial pathways. After ethanol exposure, we detected EthD-1-positive cells through-

out all pathways and generations (Figure 8B). Along the axial pathway, ethanol exposure resulted in EthD-1-positive cells in a linear pattern of distribution (Figure E10), whereas bifurcation sites off the axial pathway into major daughter branches (areas of greatest airflow turbulence) showed the greatest amount of EthD-1 staining. Daughter branches also showed diffusely scattered EthD-1-positive staining after ethanol exposure. Gaps or voids within the epithelial layer were never found after ethanol exposure. When naive rat airways were examined, we found occasional single EthD-1-positive cells within all distal pathways (generations 22–25), but none within the proximal pathways (Figure 8A). No defects within the epithelial layer were seen in naive rats. When assessing the trachea, diffuse red staining was noted within the mid-tracheal epithelium after 5% CEES exposure at 18 hours, indicating cell death (EthD-1 positive) without detachment at this stage (Figure E11C) compared with naive or ethanol-exposed tracheas (Figures E11A and E11B, respectively).

DISCUSSION

Our study demonstrated that airway casts produced after inhalation of the SM analog CEES were composed of abundant fibrin, a plasma-derived protein, and that the casts were consistently found only within the proximal half of the conducting airways (generations 2–15 from the total of 25 airway generations microdissected routinely). In addition, we showed that other plasma-derived proteins such as IgM also appeared within the airway lumen, indicating the presence of an early vascular insult leading to increased permeability. With the use of monastral blue pigment, we identified the bronchial circulation to be directly involved in the vascular leakage and, thereby, formation of casts. Injured bronchial vessels caused extravasation of plasma components into adjacent regions, which then appeared within airways. Once within airways, plasma components organized into casts capable of causing obstruction and compromising respiratory function.

Obstructive cast formation, often referred to as plastic bronchitis or cast bronchitis, is not unique to sulfur mustard exposure. Airway casts have been found in children with congenital heart diseases particularly after Fontan procedures (30, 31), as well as in patients with asthma (32–34), cystic fibrosis (35), sickle cell disease with acute chest syndrome (36), and allergic and infectious pulmonary states (37), and after burns or smoke inhalation injury (38–43). Although plastic bronchitis is not common, it is commonly fatal (44). Mortality rates reported for congenital heart disease-associated casts is 15–50% because of complete airway occlusion (45). For casts due to inhalation burn injury, mortality reaches 20–30% (43). Reports of fatal plastic bronchitis in children with asthma have also been published (32), but with lesser frequency. The composition of casts (fibrin vs. mucin) has been used as a guide to their prognosis and treatment. The Seear classification system (30) was developed to classify casts into two types: type I (fibrin casts with abundant inflammatory cells, particularly eosinophils) and type II (mucin casts with minimal cellularity). The presence of type I, or predominantly fibrin casts, results in a more ominous course, as seen with congenital heart disease, inhalational burns, and also in asthma. In our model, casts contained abundant fibrin. Although some airway epithelial cells and scant inflammatory cells were present in the “early casts” at 18 hours, no eosinophils were found. By 72 hours, increased numbers of neutrophils appeared, with the addition of newly deposited collagen and with the appearance of spindle cells (fibroblasts or myofibroblasts). A potential explanation for the relative paucity of inflammatory cells within the early casts in our model was

Figure 8. Confocal microscopic analysis of central airways (axial pathway generation 5) using double staining for live (green) and dead (red) cells, as described in METHODS, after 2-chloroethyl ethyl sulfide (CEES) inhalation (18 h). Rats were (A) naive, (B) exposed to diluent (ethanol), or (C) exposed to CEES (5%). “Gaps” in green staining of airway epithelium (arrow) were routinely noted in CEES-exposed proximal and distal airways.

that 18 hours may be an insufficient amount of time for an extensive inflammatory response to be fully manifested within the airway lumen. Indeed, analysis of BALF at 18 hours revealed an only minimal increase in absolute number of neutrophils over ethanol, further demonstrating a present but subdued inflammatory response within the airways. Whereas casts from asthma, congenital heart disease, or cystic fibrosis can take several days or weeks to form, cast formation in our model was rapid (by 18 h) after inhalation exposure. This timing of cast formation in the SM model resembles most closely that seen after burns and smoke inhalation (39, 46, 47), extensively studied in sheep (41), where casts were formed within 24 hours of injury (39). Although that model is similar to ours, it differs in three distinct aspects. First, casts after burns and smoke inhalation contain not only fibrin but also eosinophils and mucus (39). Second, casts after burns and smoke inhalation

contain extensive sloughed epithelium (39, 48), which was much less extensive in our model, appearing as distinct voids within the epithelial surface. And third, the distribution of casts throughout the tracheobronchial tree in burns and smoke injury is from the very proximal trachea down to the level of the most distal bronchioles (47), which was not the case with CEES inhalation, wherein tracheal casts were absent.

Intrapulmonary conducting airways spanning from immediately past the carina to the level of the terminal bronchioles at airway generation 15 (49) were the only locations in which casts were found after SM analog exposure. Microdissection of both fixed and unfixed lungs showed this similar distribution, where immobile casts were firmly attached to the surrounding epithelium in several locations along the bronchi. The respiratory bronchioles, alveolar ducts, and alveoli were free of casts, as was the trachea. Although the aerosol particle size (mass median diameter, 0.6–0.7 μm) would predict strong deposition even to the level of the alveoli, there was remarkably little evidence of injury to distal lungs noted by light microscopy, with no evidence of fibrin deposition within the lung parenchyma. By contrast, fibrin deposition was noted via IHC methods within the peribronchovascular space of airways containing plastic casts. Patchy epithelial sloughing was also observed via confocal microscopy, mainly within the mainstem bronchi and terminal bronchioles, but also along the distal trachea and respiratory bronchioles where neither casts nor fibrin deposition was seen. Although the exact mechanism for this epithelial sloughing has not yet been elucidated, we demonstrated that CEES inhalation injury is at least in part dependent on reactive oxygen and/or nitrogen species (50). In addition, direct damage to the epithelial cytoskeleton may occur shortly after contact with the inhaled CEES compound (our unpublished data), which may not be visible via light microscopy. Whereas sloughed epithelium was noted throughout the entire airway at 18 hours, fibrin cast formation and peribronchial fibrin deposition were not. Therefore, loss of epithelial integrity is likely not the sole cause of cast formation, albeit it may be a contributing factor. Any degree of damage to the epithelium, gross or microscopic, will expose the underlying submucosal and bronchial blood vessels, potentially facilitating entry of CEES or its downstream reactive species into the local circulation, thereby facilitating injury and increased vascular permeability. Indeed, when we employed monastral blue labeling to survey for highly permeable injured vessels, we successfully localized vascular injury to the entire bronchial plexus. Interestingly, the distribution of the bronchial circulation corresponded to the distribution of cast formation and peribronchovascular fibrin deposition (noted via IHC) along the tracheobronchial tree. Together, these findings suggest that airway epithelial injury may extend to involve the underlying bronchial circulation, leading to extravasation of plasma contents such as fibrin, and activation of the clotting cascade within the airways. Although adaptive benefits of increased vascular permeability might include recruitment of inflammatory mediators and coagulation factors designed to help in repair, we believe that the increased vascular permeability occurring after SM analog inhalation is excessive, potentially contributing to deleterious acute and chronic respiratory effects seen after exposure. Therefore, we next focused on the effects of this increased bronchial vascular permeability, particularly as it relates to leakage of fibrin and other plasma-derived proteins into the airways before cast formation.

The significant increase in plasma-derived proteins within the BALF of rats exposed to the SM analog CEES indicated a vascular injury occurring early after exposure. A concentration- and time-related increase was seen between groups in BALF fibrin, total protein, and high molecular weight IgM. Although

significant increases were noted, we suspected that the actual levels of these components in CEES-exposed BALF were likely underestimated, as casts were not included in the BALF analyzed. The increased levels of these proteins detected relative to the diluent-exposed group indicated a substantial vascular alteration allowing the leakage of these components out of the intravascular compartment. Evans blue dye injection *in vivo* allowed us to confirm the vascular leakage by noting (via microdissection) blue-labeled albumin extravasation into the peribronchovascular region from distal trachea to terminal bronchiole (airway generation 17), corresponding to the bronchial circulation. Within these regions, histological examination revealed edema formation and immunohistochemistry revealed fibrin deposition. By 72 hours after exposure, substantial spindle cells (potentially fibroblast or myofibroblast) were noted within the peribronchovascular regions, where a large amount of fibrin was noted earlier, at 18 hours. Both mature and immature collagen deposition was seen in these areas (51), particularly within the subepithelial interstitium of the submucosa, as well as within the surrounding regions. The pattern of collagen deposition seen 72 hours after CEES injury, together with the fibrinous obstructive cast formation with spindle cell invasion, resembles that seen in the early stages of bronchiolitis obliterans (52). Although bronchiolitis obliterans is a known late complication after SM injury (1, 13, 16), its pathogenesis remains unclear. We believe that our findings of acute epithelial, submucosal, peribronchial, and bronchial vascular injury with subsequent obstructive cast formation and collagen deposition within the intrapulmonary conducting airways may play an integral part in the development of bronchiolitis obliterans after SM inhalation. Further studies focusing on chronic lung injury model after SM exposure will be necessary to understand the evolution of this process.

The extensive involvement of the bronchial circulation in cast formation after inhalation injury has been seen and studied extensively in the sheep burn and smoke inhalation model (38, 40). Although SM injury differs in several respects, the similarities of early bronchial circulation involvement in cast formation seen in both models lead us to speculate that some of the underlying mechanisms could be similar. It has been proposed that three main events occurring after burn and smoke inhalation lead to early cast formation (38). First, abundant nitric oxide production appears to lead to increased blood flow into the bronchial circulation (38, 53), followed by an increase in activated neutrophils that bind to the endothelium of the bronchial vessels. Thus, reactive oxygen and/or nitrogen species might be involved. Activation of adherent neutrophils could then lead to increased vessel wall permeability (38, 54), leading to extravasation of plasma components involved in coagulation within the airway lumen. Simultaneously, the extrinsic coagulation cascade may become activated with an increase in expression of tissue factor on pulmonary epithelial cells and alveolar macrophages (38, 55), allowing cast formation to be initiated. In our model, markers of inflammation appear after cast formation has begun, and only modestly. Four hours after CEES exposure, when the first signs of cast formation become evident, BALF neutrophils were scant and lung homogenate myeloperoxidase levels were virtually unchanged from control levels. At the same time, highly permeable bronchial vessels were abundant (as per monastral blue labeling), the presence of fibrin within the peribronchovascular space was pronounced, and BALF β -fibrin and protein levels were significantly elevated. By 18 hours after CEES exposure, when casts were fully formed a more pronounced inflammatory response was evident. For this reason, we believe that although inflammation was most likely a potentiator of CEES-induced airway injury, it was not the primary cause of cast formation. We have shown that

oxidant injury is an important feature of CEES-induced airway injury, demonstrating strong attenuation of BALF plasma protein levels (IgM, protein) after 5% CEES exposure by administering the catalytic antioxidant compound AEOL 10150 (50). The role of oxidant injury in cast formation after CEES inhalation will require further investigation.

Several therapeutic strategies to limit cast formation have been studied in other causes of plastic bronchitis, especially in the burn and smoke inhalation model. The main target for these therapies has been the coagulation cascade, using anticoagulant (i.e., heparin) and fibrinolytic (i.e., tissue plasminogen activator) strategies to reduce morbidity and mortality (31, 32). These same studies have yet to be performed after inhalation injury from SM or its surrogate, CEES. Our rat model using the SM analog CEES is a useful model for conducting such therapeutic and mechanistic studies.

In summary, we have shown that inhalation of high concentrations of the SM analog CEES in rats reliably produces airway-occlusive casts similar to those noted in the literature to cause fatal obstruction in patients exposed to SM. We demonstrated increased permeability of the bronchial circulation developing early after CEES exposure, causing leakage of plasma proteins, including abundant fibrin(ogen) into airways. The resulting airway luminal casts formed then organize within 3 days of exposure, with the appearance of early histopathologic changes suggestive of bronchiolitis obliterans. This study demonstrates and confirms the usefulness of an approach combining airway microdissection and confocal microscopy using dual vital dye staining, as originally described in ozone injury (24, 25), for localizing and characterizing airway injury due to toxic inhalation and for determining disease pathogenesis. In this study, we demonstrated a rat model for airway cast formation after inhalation of the SM surrogate, CEES. This model will be useful in future studies further assessing mechanisms of cast formation, potential progression to bronchiolitis obliterans, and therapeutic interventions to limit both processes (50).

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