

Toluene diisocyanate produces an increase in airway tone that outlasts the inflammatory exudation phase

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Summary

Toluene diisocyanate (TDI) is a causative agent in occupational asthma. Through an oral catheter TDI, 0.03 μ l, dissolved in 0.02 ml olive oil, was superfused on the tracheobronchial mucosa of anaesthetized guinea-pigs. TDI induced plasma exudation into both airway tissue and lumen (peak effect: 5 hr; duration $\approx$ 17 hr). Light microscopy examinations demonstrated that the epithelium was not disrupted by this process (and that microvessels are abundant just beneath the epithelium). At days 6 and 21 after exposure to TDI PAS-positive cells were increased, but no other histological alterations were found. Also, the occurrence of peptide-containing nerve fibres was not altered by TDI. After TDI-exposure the airway smooth muscle tone was elevated as examined *in vitro* at base-line and at concentration-response to carbachol. The largest increases in tone were recorded 21 days after exposure to TDI. The abnormally large tone was not associated with an increased thickness of the smooth muscle layer nor was it associated with reduced effects of either β_2 -agonist (terbutaline) or xanthine- (theophylline) relaxants. It is concluded that TDI-induced plasma exudation into guinea-pig airways occurs for 17 hr without disrupting the epithelial lining and without causing major changes in the airway peptidergic innervation. Both the airway tone, and the number of mucous cells, are increased for at least 3 weeks after exposure to TDI.

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Introduction

Toluene diisocyanate (TDI), widely used in plastic and paint industries, is a significant cause of occupational asthma [1–3]. Two unsaturated isocyanate groups make TDI a highly reactive chemical capable of producing complex inflammatory changes in the airway mucosa [4].

It is an unresolved question whether the airway smooth muscle contractility is increased or normal in asthma [5]. McKay and Brooks have reported that guinea-pig tracheal preparations, obtained 20 hr after a series of exposures to TDI were significantly more responsive to carbachol than controls [4]. This observation, suggesting that the airway smooth muscle itself was affected by TDI, has not been confirmed. Indeed, this particular possibility of an induced smooth muscle abnormality is not discussed in those further studies from the same laboratory,

which demonstrate increased *in vivo* histamine responsiveness in guinea-pigs previously exposed to TDI [6].

We have previously demonstrated that superfusion of the guinea-pig tracheal mucosa with 0.003–0.03 μ l of TDI produces a dose-dependent and sustained inflammatory response, recorded as plasma exudation into the airway lumen [7]. At the 5 hr peak response the number of leucocytes was increased in the lumen [8]. However, a role of leucocytes in the sustained exudative response to TDI is in doubt: both hydroxyurea- and cyclophosphamide-treatments depleted the animals of leucocytes, but only hydroxyurea reduced the plasma exudation effect [8]. In the present study we have repeated the measurements of exuded plasma tracers, to determine the time course of ongoing mucosal/submucosal inflammatory exudation after a single exposure to TDI. We have then examined base-line tone as well as contractile and relaxant responses in tracheal rings obtained during and after the inflammatory exudative response to TDI. Further, we

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have examined the effect of TDI on the mucosa/submucosa including effects on the density of mucous cells and the occurrence and distribution of peptide-containing nerve fibres and histamine-containing cells (mast cells).

Materials and methods

Administration of TDI in vivo and sampling

Animals. Male guinea-pigs, showing a normal weight gain during an observation period of 7–10 days and weighing about 350 g, were used. On the experimental day, the animals were starved for 3 hr and anaesthetized with a 3:2 mixture of ketamin (50 mg/ml), and xylazin (20 mg/ml), 0.1 ml/100 g intramuscularly.

Superfusion of the tracheal mucosa with TDI in vivo [7]. The animals were put in a head up tilted position [7]. A plastic tubing (OD 0.61 mm) was introduced into the tracheal lumen via the mouth by aid of a fine lachrymal duct cannula. After retraction of the cannula, the tip of the catheter was placed about 0.5–1 cm below the larynx (measured by markings on the catheter). After a resting period of 10–15 min, the tracheal mucosa was superfused with 0.02 ml of the vehicle (olive oil) with or without TDI 0.03 μ l at a constant rate (Sage servo pump) during 1 min. After treatment and withdrawal of the catheter the animals were kept in the tilted position for 5 min. Then the animals were put in cages and placed on heating pads to maintain body temperature.

The distribution of superfused olive oil within the tracheobronchial tree was visualized by superfusing olive oil saturated with bromchresolgreen ($\approx$ 2 mg/ml, 0.02 ml/min). The animals were killed 10 min later and the trachea including larynx and lungs were excised *in toto*. The trachea and main stem bronchi were cut open ventrally. The mucosal surface in the trachea and main stem bronchi, as well as upper intrapulmonary bronchi (at the hilar levels), were covered with bromchresolgreen. There were no signs of green colour in the more peripheral parts of the lungs.

Study of exudation effects of TDI. At 5 and 17 hr and 6 days after TDI exposure the animals were killed. Fifteen minutes prior to termination the plasma macromolecular tracer fluorescein isothiocyanate conjugated dextran (FITC-dextran) (MW 156 kD, 5% solution, 0.2 ml/100 g) was given intravenously via an ear marginal vein to conscious animals.

Termination of experiment and sampling [7]. The experiment was terminated by administration of 0.15–0.2 ml of pentobarbital (Mebumal 60 mg/ml) intravenously. The chest was opened and the animal was exsanguinated by

sectioning of the still beating heart and the vena cava. Mixed arterial-venous blood samples were collected in pre-weighed plastic tubes. Some of the blood samples (after addition of heparin) were centrifuged (500 g for 10 min) for separation of plasma and red blood cells.

Tracheal lavage [7]. The trachea and lung, including larynx, and heart, were excised *in toto*. The laryngeal cut edges were dried with a cell cotton cloth. After a brief rinse in saline in order to remove excess blood from the surface, the whole preparation was put in a tilted position with the larynx upwards. Each lobar bronchus was ligated at the hilar level. A slightly bent plastic tubing (PE50) was introduced through a small incision (previously dried with a cell cotton tip) into the left principal bronchus and secured by a ligature. The preparation was then mounted vertically with a small clamp attached to the larynx. The trachea was gently lavaged with 0.25 ml saline 3–4 times during 1 min. The lavage fluid was infused to a level well below the region where the tip of the intratracheal catheter had been placed. The lavaged portion could be well controlled because the fluid level was clearly visible through the thin illuminated tracheal wall. The tracheal lavage fluid and specimen of intrathoracic tracheal tissue that had been lavaged were placed in pre-weighed tubes.

Analyses

The content of FITC-dextran in the tracheal tissue samples was eluted by adding 2 ml sodium phosphate buffer, pH 7.5, followed by shaking for 15 hr and centrifugation. Dilutions of the supernatants thus obtained, as well as of plasma samples, tracheal lavage fluids, and FITC-dextran control solutions were analysed in a fluorimeter (Perkin-Elmer LS-5).

Plasma corresponding to FITC-dextran in samples was calculated as follows:

$$\frac{\text{FITC-dextran in lavage and tissue (50 mg)}}{\text{FITC-dextran in 1 g plasma}}$$

Study of airway smooth muscle responsiveness in vitro

In a preliminary study we observed that the TDI-treatment was followed by increased tracheal tone. Both the spontaneous tone and the carbachol-induced tone were thus elevated 6 days after TDI-exposure (data not shown). In this study animals were killed (by a blow to the head) 5 and 17 hr, and 6 and 21 days after the superfusion with TDI 0.03 μ l. The intrathoracic part of the trachea was excised. One segment was used for the *in vitro* evaluation and the other part was immersed in fixative for histology and immunocytochemistry (see below).

Table 1. Recovery of plasma (FITC-dextran) in lavage and tracheal tissue after TDI superfusion ($n=6$), mean $\pm$ s.e.m.

	Lavage (μ l)	Tracheal tissue (μ l/50 mg)
Control		
5 hr	0.14 ± 0.04	1.87 ± 0.19
17 hr	0.05 ± 0.02	2.14 ± 0.18
6 days	0.12 ± 0.03	1.98 ± 0.21
TDI		
5 hr	$4.40 \pm 0.52^{***}$	$5.29 \pm 0.52^{***}$
17 hr	$0.97 \pm 0.10^{***}$	2.80 ± 0.45
6 days	0.23 ± 0.04	1.99 ± 0.51

Significantly different from corresponding control
 $***P < 0.001$, Student's *t*-test.

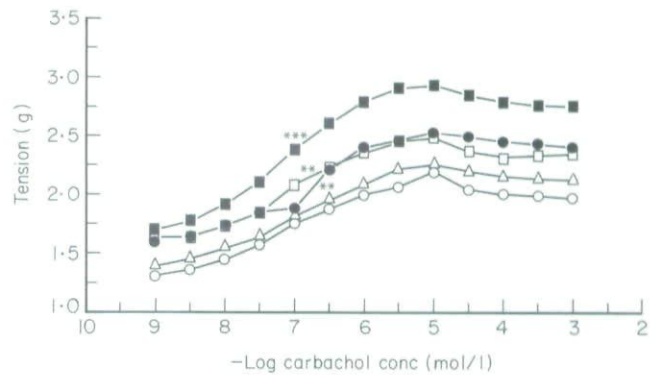
Table 2. Basal tensions and maximum carbachol-induced tensions in grams in guinea-pig tracheal ring preparations *in vitro*, mean $\pm$ s.d.

	<i>n</i>	Basal tension	Maximum tension
Control (mean)	24	1.26 ± 0.31	2.09 ± 0.49
Control (5 hr)	6	1.17 ± 0.21	1.72 ± 0.20
TDI (5 hr)	6	1.38 ± 0.33	$2.23 \pm 0.47^*$
Control (17 hr)	6	1.50 ± 0.40	2.27 ± 0.38
TDI (17 hr)	6	1.60 ± 0.46	2.49 ± 0.67
Control (6 days)	6	1.15 ± 0.35	2.08 ± 0.64
TDI (6 days)	6	1.58 ± 0.51	2.54 ± 0.37
Control (21 days)	6	1.22 ± 0.13	2.30 ± 0.49
TDI (21 days)	6	$1.64 \pm 0.39^*$	$2.94 \pm 0.50^*$

Significantly different from corresponding control $*P < 0.05$, Student's *t*-test.

A segment comprising two adjoining cartilage rings from the intra-thoracic part of the trachea, just above the bifurcation, was cut out. A single open tracheal ring was then obtained by cutting through the cartilage opposite the muscle. This open ring preparation was mounted in a jacketed, temperature-controlled (37°C) 25 ml organ bath containing Krebs' solution of the following composition (mm): NaCl 118.0, KCl 4.6, CaCl_2 2.5, MgSO_4 1.15, NaHCO_3 24.9, KH_2PO_4 1.15 and glucose 5.5, with a pH of 7.4 and aerated with 5% CO_2 in oxygen.

Changes in muscle tension were recorded isometrically

**Fig. 1.** Concentration-response lines to carbachol in control preparations ($\circ$) and in preparations obtained at four different times after TDI treatment; TDI 5 hr (Δ), TDI 17 hr ($\square$), TDI 6 days ($\bullet$) and TDI 21 days ($\blacksquare$). Each point is a mean value of isometric tension (g) changes. Absolute standard deviations for all measurements varied from 0.30–0.67 g. Significant shifts of the lines (parallel line assay) are indicated ($*** = P < 0.001$; $** = P < 0.01$ compared to the control curve).

by means of strain gauge transducers (Grass FT03). All measurements were recorded on a Grass polygraph. The initial mounting tension was set at 0.5 g. Preparations were allowed to stabilize for at least 30 min with repeated washings until stable recordings of tone were achieved. The cumulative concentration-response relationship for carbachol was determined by increasing stepwise the concentration in the bath (range 10^{-9} to 10^{-3} M) until no further effect was achieved by two consecutive concentrations.

After the cumulative concentration-response relationship of carbachol had been determined, the trachea was washed and allowed to return to its base-line tone. The terbutaline or theophylline was added in cumulatively increasing concentrations (10^{-9} to 10^{-4} M and 10^{-7} to $3 \cdot 10^{-3}$ M, respectively) to achieve maximal effect of either drug. After the trachea had been washed and the tone had returned to baseline, it was contracted with carbachol $5.4 \cdot 10^{-7}$ M (corresponding to 70% of the maximal effect of carbachol). Concentration-response to terbutaline or theophylline was then again evaluated in the presence of carbachol.

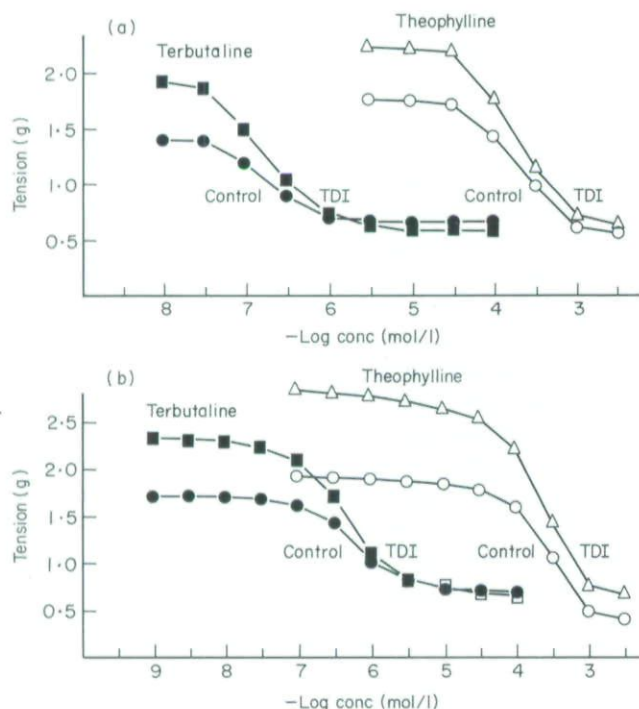
Histology and immunochemistry

Tracheal specimens from each group were fixed in Bouin's fixative, dehydrated and embedded in paraffin. Sections were cut at 4 μ m thickness, deparaffinized in xylene, hydrated and stained with periodic acid-Schiff reagent (PAS) and examined for the presence of seromucous cells. For glycoprotein-containing structures PAS is

Table 3. — Log EC₅₀-values (mol/l) for carbachol-contraction and relaxation with theophylline or terbutaline (after carbachol-precontraction) in guinea-pig tracheal ring preparations *in vitro* (study II), mean $\pm$ s.d.

	<i>n</i>	Carbachol	<i>n</i>	Theophylline	Terbutaline
Control (mean)	24	7.24 $\pm$ 0.36	12	3.66 $\pm$ 0.12	6.24 $\pm$ 0.16
Control (5 hr)	6	7.46 $\pm$ 0.62	3	3.55 $\pm$ 0.03	6.29 $\pm$ 0.17
TDI (5 hr)	6	6.93 $\pm$ 0.29	3	3.88 $\pm$ 0.17	6.54 $\pm$ 0.04
Control (17 hr)	6	7.07 $\pm$ 0.26	3	3.59 $\pm$ 0.08	6.24 $\pm$ 0.22
TDI (17 hr)	6	7.00 $\pm$ 0.16	3	3.64 $\pm$ 0.06	6.44 $\pm$ 0.20
Control (6 days)	6	7.18 $\pm$ 0.16	3	3.76 $\pm$ 0.08	6.09 $\pm$ 0.11
TDI (6 days)	6	7.00 $\pm$ 0.20	3	3.55 $\pm$ 0.06*	6.12 $\pm$ 0.21
Control (21 days)	6	7.24 $\pm$ 0.18	3	3.72 $\pm$ 0.11	6.32 $\pm$ 0.11
TDI (21 days)	6	7.16 $\pm$ 0.10	3	3.76 $\pm$ 0.08	6.34 $\pm$ 0.02

Significantly different from corresponding control * $P < 0.05$, Student's *t*-test.

**Fig. 2.** Mean relaxant effects of theophylline and terbutaline in baseline (a) and carbachol-contracted (10^{-7} M) (b) tracheas obtained from control animals and animals previously (21 days) treated with TDI. Theophylline control (○), theophylline after TDI-treatment (Δ), terbutaline control (●) and terbutaline after TDI-treatment (■). Absolute standard deviations for all measurements varied from 0.23–0.56 g. Despite the larger tone in TDI-treated preparations the relaxant drugs were as potent and efficacious as in controls.**Table 4.** Number of PAS-positive mucous cells per unit length (0.7 mm) of sectioned guinea-pig mucosa ($n = 10$ each group), mean $\pm$ s.e.m.

	Control	TDI 0.03 μ l
6 days after exposure	28.2 $\pm$ 3.3	42.6 $\pm$ 2.3**
21 days after exposure	22.7 $\pm$ 3.3	46.4 $\pm$ 6.1**

Significantly different from corresponding control ** $P < 0.01$, Student's *t*-test.

a specific stain for fucose and has been used for quantification of secretory cells in the respiratory tract [12]. The PAS-positive cells of the epithelium were counted (in a single-blinded way) in sections cut perpendicularly to the mucosal surface so that the entire height was visible. Consecutive sections were discarded to avoid repeated counting of the same cells. Every third section was used. The cells were counted per visual field (diameter 0.7 mm, 10 sections per animal, four visual fields covering the total circumference per section, magnification $\times 250$) and the cell number expressed per unit length of sectioned mucosa. The thickness of the tracheal smooth muscle was measured in a single-blinded way by using a micrometre scale inserted in the visual field. The thickness was measured on the midpoint between the edges at the tracheal cartilage.

Additionally, specimens were fixed by immersion in an ice-cold mixture of 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) overnight and thoroughly rinsed in Tyrode solution containing 10% sucrose. They were then

frozen on dry ice and sectioned at 10 μm thickness in a cryostat. The sections were processed for the immunocytochemical demonstration of substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and histamine [9]. The SP antiserum (code SP8, gift from Dr P. C. Emson, Cambridge, U.K.) was used in dilution 1:320. The CGRP antiserum (code 8427, Milab, Malmö, Sweden) was used at a dilution of 1:1280. The VIP antiserum (code 7852, Milab, Malmö, Sweden) was diluted 1:640 and the histamine antiserum (code 8431, Milab, Malmö, Sweden) 1:1280. The sections were exposed to either of the antisera for 24 hr in a moist chamber. The site of the antigen-antibody reaction was revealed by application of FITC-labelled antibodies against immunoglobulin G (Milab) at a dilution 1:320 for 1 hr at room temperature. Control sections were exposed to primary antiserum that had been pre-absorbed with an excess amount of the antigen (10–100 μg of synthetic peptide [Peninsula Labs, Belmont, U.S.A.] or histamine [Fluka] per millilitre diluted antiserum). The neuropeptide antibodies used have previously been shown to not cross react with other known neuropeptides [13–15]. Nevertheless, cross-reaction with still unknown peptides or proteins containing amino acid sequences recognized by the different antisera cannot be excluded. It is appropriate, therefore, to refer to the immunoreactive material as SP-like, CGRP-like and so on. For simplicity, however, the shorter terms SP, CGRP and so on are used henceforth. The sections were examined in a fluorescence microscope fitted with appropriate filter setting for viewing FITC.

Calculations and statistics

Data are presented as means and standard errors. Linear regression analyses were performed at three or four points of measurement (log dose versus effect) on the linear part of the concentration-response curves and any vertical shift of parallel lines was determined by the regression analysis. From the equation of each regression line we obtained an EC_{50} -value (the concentration producing 50% of the maximal effect). EC_{50} -values were log-transformed for analyses and geometric mean values obtained. Significant differences amongst means were examined with Student's *t*-test for unpaired observations (* = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$). No correction for multiplicity was made.

Changes in absolute tension induced by carbachol, theophylline or terbutaline were also calculated.

Chemicals

Ketamin (Ketalar, 50 mg/ml, Parke-Davis, USA); xylazin (Rompun vet, 20 mg/ml, Bayer, Germany); pentobarbital (Mebumal vet, 60 mg/ml, ACO Sweden).

Fluorescein isothiocyanate conjugated dextran (FITC-dextran, MW 156 kD, Bioflor, Sweden).

Toluene diisocyanate (80% 2,4-TDI, 20% 2,6-TDI, density 1.2, Merck, Germany) diluted in olive oil in a concentration of 1.5 μl TDI/ml olive oil.

Carbamylcholine chloride (Carbachol, Sigma, USA), theophylline (20 mg/ml, Draco, Sweden) and terbutaline sulphate (0.5 mg/ml, Draco, Sweden) diluted in a 0.9% sodium chloride solution.

Results

Plasma tracers in lavage and tracheal tissue after TDI superfusion in vivo

In confirmation of earlier work [7] the TDI-induced plasma exudation peaked at 5 hr. At 17 hr the exudation was small but still significant into the lumen but not into the tissue (Table 1). Six days after exposure to TDI the inflammatory plasma exudation had ceased (Table 1).

Airway smooth muscle responsiveness

The preparations developed a tone *in vitro* which was already stable 20–30 min after mounting. The TDI-treated tracheas had a higher spontaneous tone than control preparations ($P < 0.01$) (Table 2) and the maximal carbachol-induced tensions were similarly increased after TDI exposure ($P < 0.01$) (Table 2). There was a significant upward shift of the C/R lines to carbachol 17 hr ($P < 0.01$), 6 days ($P < 0.01$) and 21 days ($P < 0.001$) after the TDI treatment (Fig. 1). The potency of carbachol, as determined by EC_{50} -values, was not affected (Table 3).

The relaxant potencies of theophylline and terbutaline were not different in control and in TDI-treated preparations (Table 3, Fig. 2) in the presence or absence (data not shown) of carbachol. Terbutaline was a less potent relaxant in carbachol-contracted preparations ($-\log \text{EG}_{50} = 6.24 \pm 0.16 \text{ mol/l}$) than of those with spontaneous tone ($-\log \text{EC}_{50} = 6.70 \pm 0.11 \text{ mol/l}$).

Light microscopy examination (PAS-stained sections) did not reveal any differences in the general histology of the tracheal wall between control or any of the TDI-exposed tracheal specimens (Table 4). A prominent feature was the abundance of subepithelial microvessels (Fig. 3). The height of the epithelium was not changed. The number of epithelial PAS-positive cells (which stained bright reddish) was significantly ($P < 0.01$; $n = 10$) increased in density in the tracheal specimen exposed to TDI 6 and 21 days earlier compared to control (Table 4). At shorter times (5 hr, 17 hr) these cells were not increased.

There was no difference in the distribution of the

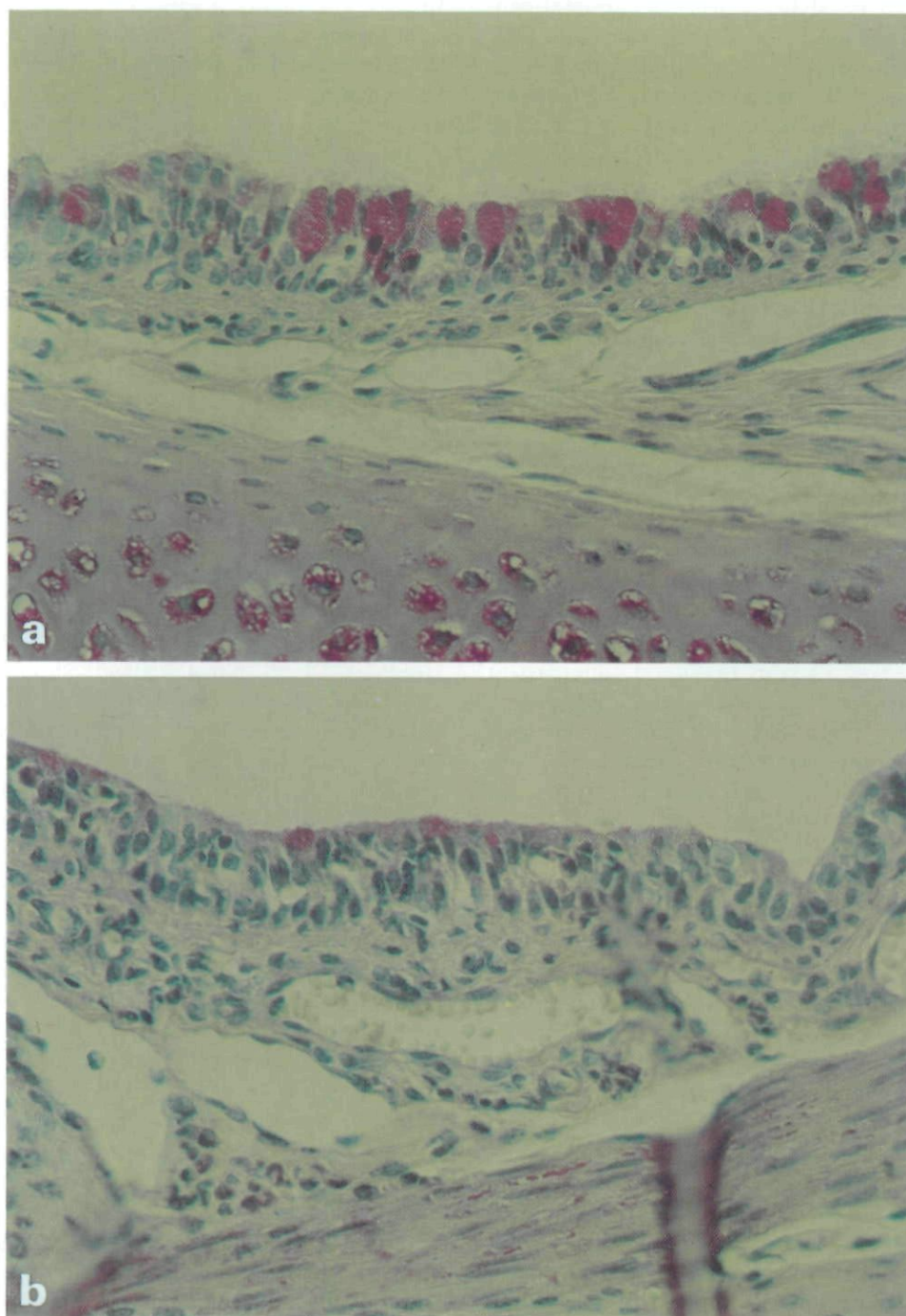


Fig. 3. Guinea-pig tracheal wall. TDI-treated (a) and control (b). Sections stained with PAS. Numerous PAS-positive cells in the respiratory epithelium of TDI-treated animals; they are only moderate in number in the control animal. Subepithelial microvessels are prominent in the guinea-pig trachea. Magnification $\times 250$.

peptide-containing nerve terminals or of histamine containing cells. The latter type of cells occurred only rarely. Both in controls and TDI-treated specimens SP-containing nerves were found in the epithelium, the subepithelial and smooth muscle layers (Fig. 4). CGRP-containing nerve fibres were somewhat more abundant but had the same distribution pattern as the SP-containing fibres. VIP-containing nerve fibres were not present in the epithelium but were mostly localized to smooth muscle and vasculature. The mean thickness of the smooth muscle layer was $74 \pm 12 \mu\text{m}$ in controls and $75 \pm 11 \mu\text{m}$ in tracheal specimens obtained 3 weeks after the TDI exposure (mean $\pm$ s.d.; $n = 10$).

Discussion

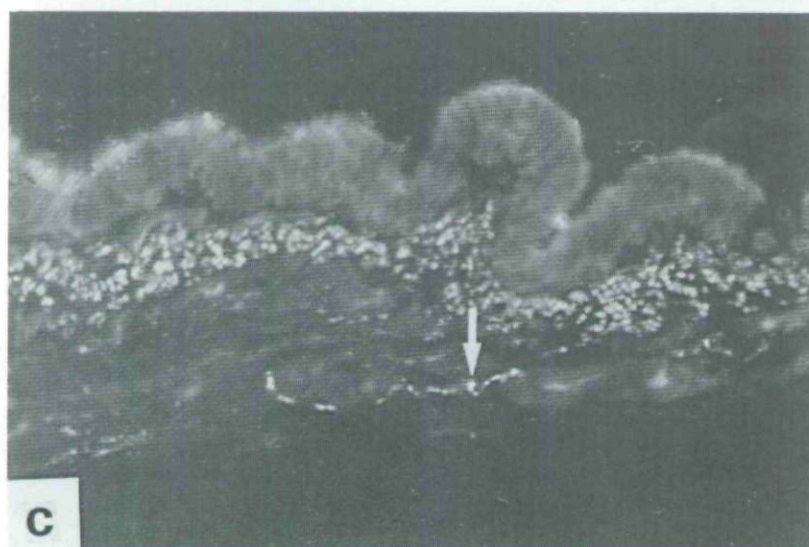
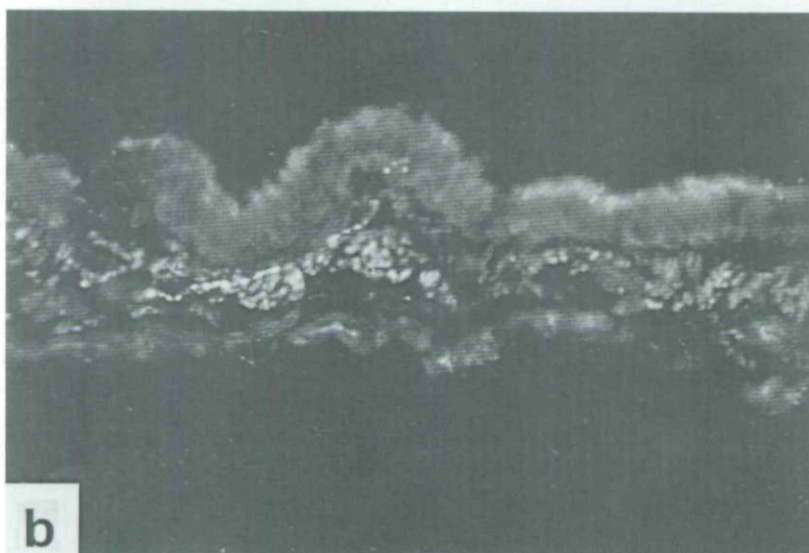
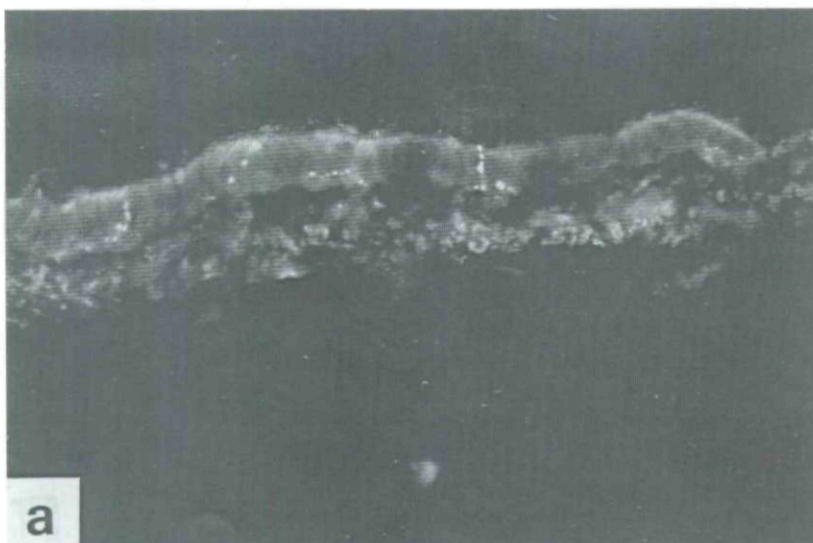
Superfusion of the guinea-pig tracheal mucosa with TDI produces a characteristic plasma exudation response. Within 10 min after a brief exposure to TDI, plasma macromolecules have extravasated into the airway mucosal/submucosal interstitial space and have also passed across the epithelium. There is an abundance of subepithelial microvessels (see Fig. 3) from which exudation occurs and the epithelium is a poor barrier to plasma exudates approaching from beneath [10] (see below). Plasma exudation is a cardinal sign of inflammation and has been proposed to be a multipotential pathogenic factor in asthmatic airways [11]. A previous study has demonstrated that the amount of luminal plasma tracers is well correlated with the amount exuded into the tissue interstitial space suggesting that plasma on the mucosal surface can quantitate an ongoing mucosal/submucosal inflammation [7]. In fact, due to low background values, the measurement of plasma macromolecular tracers in samples of mucosal surface liquids is a particularly sensitive inflammatory index [7,10]. This view is supported by the response at 17 hr in this study, where FITC-dextran was significantly elevated in the lumen, but not in the tissue. FITC-dextran is probably a suitable marker of plasma exudation. In contrast to albumin it may not be actively secreted into the lumen [16].

The present data are consistent with a peak inflammatory response to TDI at 5 hr, which has declined considerably by 17 hr. Fabbri *et al.* [17] have demonstrated that a challenge with TDI, in subjects with TDI-asthma, increases albumin levels in bronchial lavage fluid obtained prior to and during a late phase response. In consideration of the marked change from 5 hr to 17 hr in this study, the vascular/mucosal exudation probably has normalized within a day or two. Data obtained 6 days after exposure confirmed that the TDI-induced plasma exudation was fully reversible.

The present technique of superfusing the airway with TDI is different from exposures to TDI vapours, but it may have experimental advantages. Controlled doses can be applied and the application is selective, sparing both nasal passages and the most peripheral airways. Olive oil in itself is without exudative effect [7] and it distributes to the large tracheobronchial airways (this study) as do superfusions with saline. The dose of TDI employed in this study has previously been shown to produce a consistent, submaximal, inflammatory response [7]. The maximum airway exposure to TDI during a working day may be about $15 \mu\text{g}$ TDI/kg body weight [18]. This figure would be larger in those workers, who have been exposed to accidental spills of TDI and who are at particularly high risk of developing asthma [1,19]. In the present study the guinea-pigs were exposed to about $0.1 \mu\text{g}/\text{kg}$ of TDI, but the exposure was much concentrated in time and the exposed mucosal surface was confined to the large tracheobronchial airways.

Previously, we have observed that 10 min after topical superfusion with PAF or capsaicin the epithelial lining appeared histologically intact in light and electron microscopy despite the fact that large amounts of plasma macromolecular tracers, corresponding to a plasma volume larger than the periciliary fluid volume, had been exuded into the airway lumen [20,21]. A maintained integrity of the mucosal barrier is also suggested by the observation that absorption of luminal macromolecules was not increased even during exudation of plasma [7,22]. The distinction between outward exudative and inward absorptive mucosal permeabilities is valid also for the sustained TDI-induced effects (unpublished observations by Erjefält and Persson). The passage of plasma exudate across the mucosa is probably through paracellular pathways reversibly induced by an increased hydrostatic pressure acting on basolateral aspects of the epithelial cells [23]. In this study the more sustained plasma exudation process, as induced by TDI, occurred across an epithelial lining that histologically appeared intact. This observation supports the view that luminal entry of exuded plasma is a non-injurious primary airway defence reaction [24]. It was only long after the exudation phase that the present light microscopic morphology revealed a difference between TDI-exposed tracheas and control tracheas: the number of PAS-positive cells was significantly increased 6 and 21 days after exposure. An increased number of mucous cells has previously been observed in guinea-pig airways one and 3 weeks after repeated exposure to TDI-vapours [4,25].

It has been speculated that in asthma there may be an increased density of bronchoconstricting tachykinergic nerves and a reduced density of bronchodilating VIP-like innervation. Specifically it has been suggested that TDI-



induced increase in airway responsiveness in guinea-pigs is mediated by tachykinins [26]. However, the distribution in the guinea-pig trachea of nerves containing neuropeptides, such as CGRP, tachykinins and VIP appeared not to be affected by TDI in this study. Nor could we detect any changes in the presence of histamine-containing cells.

The guinea-pig isolated tracheal ring preparations normally develop a significant spontaneous tone after mounting in the organ bath. In the presently employed single ring preparation this occurs relatively rapidly so that quite stable recordings are obtained, usually within 30 min after mounting (this study) [27]. The tone is independent of the presence of an intact epithelial lining [27] but the mechanisms involved in the generation of this tone have not been well assessed. This study demonstrated that preparations obtained after TDI exposure had developed an abnormally high tone spontaneously and in response to carbachol. Three weeks after the exposure the largest tone was recorded. McKay and Brooks [4,18] previously reported that the base-line tone of their guinea-pig tracheal preparations was not affected whereas the effect of carbachol was increased by prior *in vivo* exposure to TDI. However, it is difficult to compare the results. For example, McKay and Brooks [4] stretched their spirally cut tracheal strips to a tension of 4 g, and the tone was slightly reduced during the course of their experiment [4]. In contrast, the single open ring preparation was initially set at 0.5 g, from which level a further increased tone developed spontaneously and promptly [this study, 27–30]. The present data suggest that prior exposure to TDI produces an increased tone in airway smooth muscle tone both under base-line conditions and in the presence of carbachol. In a separate study (data not shown) where the design was blinded to the *in vitro* investigator we have confirmed the upward shift ($P < 0.001$) of the carbachol curve 17 hr ($n = 6$) and 6 days ($n = 6$) after exposure to TDI compared to control ($n = 12$). The basis for this change in smooth muscle is not known. Nothing abnormal was detected in the present histological examinations of smooth muscle including size nor was there any abnormality in the response to relaxant agents. Further studies are required to elucidate the mechanisms behind the present observations. Mapp *et al.* [31] have found that TDI 0.01–1 mM contracts smooth muscle when added *in vitro*. This is an acute response that may not be related to the present effect that is induced over a prolonged time. Also, the present effect

is recorded with smooth muscle concentrations of TDI which presumably are several orders of magnitude less than the *in vitro* levels TDI [31]. Thompson *et al.* [26] reported that 2 hr after exposure to TDI guinea-pigs exhibited increased sensitivity to the airway obstructive effect of inhaled acetylcholine. It is possible that an effect on the smooth muscle as found in this study has contributed to the increased responsiveness. However, in the acute inflammatory phase other factors, including the exudative effect, may have contributed. Thompson *et al.* [26] only assessed the total amount of Evans blue dye in the airway tissue. Hence, they could not determine whether extravasated plasma in the tissue or in the lumen were associated with the altered response to acetylcholine.

β -agonists and, to a lesser extent, xanthines have a relaxant potency that is reduced the more contacted the tracheal preparations [28,29]. These observations are consistent with the present data in control tracheas, where the EC_{50} of terbutaline was increased in carbachol-contracted preparations compared to preparations with intrinsic tone only. However, the elevated tone that was present in the TDI-exposed tracheas, both under base-line conditions and in the presence of carbachol, was not associated with reduced responses to airway relaxant drugs. These findings also show that the increased tone may not be caused by an effect of TDI on the airway smooth muscle β -receptors.

In conclusion, the present study has demonstrated that exposure of guinea-pig tracheobronchial airways to 0.03 μ l TDI induces a sustained but fully reversible inflammatory response with appearance of plasma exudate on the mucosal surface. The present light microscopy examination demonstrated that microvessels are abundant adjacent to the epithelial base and that the TDI-induced sustained luminal entry of plasma exudate has occurred without disruption of the epithelial lining. It further appeared that TDI-exposure did not affect the airway distribution of neuropeptide-containing structures. Three weeks after TDI exposure the number of mucous (PAS-positive) cells was significantly increased. Further, we observed that the tracheal tone *in vitro* was increased above control and so was the tone induced by carbachol. It cannot be excluded that the present TDI-induced alterations in airway smooth muscle tone is an important aspect of the long-term airway effects of exposure to TDI.

Fig. 4. TDI-treated guinea-pig trachea. Sections immunostained for SP. Fine varicose SP-containing nerve fibres penetrating the epithelium (a), located in the subepithelial layer (b) and in the submucosa among bundles of smooth muscle (arrow) and surrounding blood vessels (c). The occurrence and distribution of SP-containing nerve fibres were the same in control animals (illustration not shown). Magnification $\times 250$.

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