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Characterization of a Guinea Pig Model of Toluene Diisocyanate Induced-Occupational Asthma

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SUMMARY

The lungs are the most heavily utilized organs in the body. In a lifetime, the average person will breath over 300 x 10^6 L of air, a volume 5000 times greater than the volumetric intake of food or water (Phalen and Prassard, 1989). In addition to being responsible for the uptake of oxygen to maintain metabolism and the elimination of carbon dioxide produced by cellular metabolic processes, the lungs must defend against countless airborne aggressors including pollutants, allergens, viruses, bacteria and microbes. As a result of the vital role of the lung, pulmonary diseases have an enormous social and economic impact. Of these diseases, asthma is certainly the most important. Asthma has been estimated to affect 10-15% of the population of industrialized countries (Sears, 1990) and is known to be the only "preventable" disease for which morbidity and mortality are rising (Page, 1993). A common cause of asthma in industrialized countries is occupational exposure to certain chemicals, of which toluene diisocyanate (TDI) is considered one of the most important. The study of TDI-induced asthma in guinea pigs represents a very useful model for the investigation of the pathogenesis of TDI-induced occupational asthma and of asthma in general.

The objective of this research was to characterize the effects of TDI vapor exposure in the guinea pig lung, under the conditions established in our laboratory, through the correlation of inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and morphometric effects in the bronchiolar epithelium.

In this research guinea pigs were sacrificed for pharmacological, cytological, morphological and morphometric evaluations one, seven and 21 days following 5 days of 4 hour per day inhalation exposure to 3 ppm toluene diisocyanate (TDI) .

Bronchoalveolar lavage (BAL) provided evidence of a marked infiltration of leukocytes into the airways following 5 days of exposure to TDI. When compared with concurrent controls, a greater than four fold increase in total number of cells recovered in bronchoalveolar lavage fluid (BALF) was observed one day after the completion of TDI exposure. Seven days post-exposure the total number of cells recruited into BALF had decreased to approximately twice the concurrent control values while 21 days after exposure completion approximately equivalent numbers of cells were retrieved in BALF. In addition to marked infiltration of total numbers of leukocytes, BALF results

provided evidence of changes in the proportional representation of cell populations recovered from the airways of air and TDI-exposed guinea pigs. Differential cell counts were performed to determine the relative numbers of macrophages, eosinophils and neutrophils. When compared with corresponding controls, these counts indicated two, 20 and 50 fold increases in the numbers of macrophages, eosinophils and neutrophils, respectively, one day after exposure completion.

Challenge of standardized, isolated bronchial strips with non-specific agonists (histamine and acetylcholine) provided marked evidence of bronchial hyperreactivity. Responses were most pronounced the day after exposure completion. When expressed in terms of the area under the dose-response curves constructed from the summed right and left bronchial responses, the responses 1 day after exposure were approximately 3 to 4 times that observed 7 and 21 days post-exposure. Responses observed 7 and 21 days post-exposure were similar and were approximately two to three times the concurrent control values.

Qualitative microscopic evaluation of all sections obtained from guinea pigs sacrificed 1, 7 and 21 days after exposure completion were performed. When compared with air controls, histopathological changes observed 1 day following exposure completion clearly indicated epithelial damage characterized by epithelial sloughing, replacement of the normal pseudostratified, ciliated columnar epithelium by stratified cuboidal epitheliod cells lacking cilia, clear submucosal inflammation and edema. Seven days after exposure completion, the beginnings of repair were evident and were most obvious by the lack of epithelial sloughing. While submucosal inflammation was still observed, it was present to a lesser extent than was observed the day following exposure completion. Similarly, the epithelium had not yet returned to the normal pseudostratified, ciliated columnar appearance. Slides prepared from animals sacrificed 21 days after exposure completion indicated obvious epithelial regeneration and a relatively normal histopathological appearance.

Histomorphometric evaluations of one representative control and one TDI-exposed guinea pig sacrificed 1, 7 and 21 days after exposure completion were used to provide a semi-quantitative evaluation of the ratio of the volume of infiltrating cells to the submucosal surface area. The data indicated that 1 day after exposure completion the volume of the submucosa represented by infiltrating cells was approximately 3 times that the control value, while 7 and 21 days later this had decreased to approximately twice and approximately 1.5 times the control value, respectively.

The characterization of the response of the guinea pig lung to TDI vapors described in this research provides an excellent correlation between inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and morphometric changes in the bronchiolar epithelium. This research provides a solid foundation on which to continue investigations into the causes of TDI-induced asthma. This model may be used in the future to study the mechanisms of TDI-induced asthma through pharmacological intervention with the goal of abrogating or inhibiting one or more responses observed in these studies.

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RÉSUMÉ

1. OBJECTIF

Ce projet avait pour but de caractériser les effets de l'exposition aux vapeurs de toluène diisocyanate (TDI) sur le poumon de cobaye. Cette caractérisation a été démontrée par la corrélation des changements causés par l'inflammation des voies respiratoires, de l'hyperréactivité de bronchique provoquée par un agoniste ainsi que par les changements histologiques et morphologiques observés au niveau de l'épithélium bronchiolaire. Ce modèle expérimental servira de base à un projet plus vaste dans lequel on examinera les mécanismes de l'asthme provoqué par le TDI chez les travailleurs. Le cobaye est l'animal de choix pour étudier l'asthme professionnel induit par le TDI. En effet, il existe de nombreuses similitudes entre les réactions de l'organisme humain et celles de cet animal, notamment l'inflammation des voies respiratoires, l'hyperréactivité bronchique à divers bronchoconstricteurs ainsi que des réponses pulmonaires similaires à une grande variété d'agents pharmacologiques, y compris les stéroïdes et les anti-inflammatoires non stéroïdiens (Cibulas *et al.*, 1986).

2. REVUE DE LITTÉRATURE

Le poumon constitue la région de l'organisme où s'effectuent l'absorption de l'oxygène nécessaire au maintien métabolique ainsi que l'élimination du bioxide de carbone produit par le processus métabolique des cellules. Sa capacité à remplir cette fonction vient du fait qu'il est composé d'immenses surfaces où le sang entre virtuellement en contact avec l'air de l'atmosphère. Chez le mâle adulte, la surface d'absorption du poumon couvre 150 mètres carrés et est répartie dans 300 millions d'alvéoles. Un réseau de capillaires couvre environ 80-90% de cette surface afin d'assurer les échanges gazeux (Weibel, 1973). Au cours de sa vie, un individu moyen respire plus de 300 x 10^6 litres d'air, un volume 5000 fois plus grand que l'apport d'eau ou de nourriture (Phalen et Prassard, 1989). En raison du rôle vital du poumon, les maladies pulmonaires ont un énorme impact social et économique.

Étant donné sa fréquence (selon Sears, 1990, on estime que l'asthme affecte 10 à 15% de la population) et sachant qu'il s'agit d'une maladie "évitable" dont les taux de morbidité et de mortalité sont en croissance (Page, 1993), on peut en arriver à considérer l'asthme comme étant

la plus importante maladie pulmonaire. A l'heure actuelle, l'asthme est considéré comme "une affection inflammatoire chronique des voies aériennes dans laquelle plusieurs cellules jouent un rôle, dont les mastocytes et les éosinophiles" (Rapport du consensus international sur le diagnostic et le traitement de l'asthme, 1992). Cette maladie présente deux épisodes séparés d'obstruction bronchique, sans rapport de causalité. La réaction asthmatique immédiate est un épisode de bronchoconstriction qui apparaît habituellement dix à vingt minutes après une exposition à un allergène. La réaction asthmatique tardive est une augmentation retardée mais soutenue de la résistance des voies aériennes qui se manifeste habituellement trois à huit heures après une exposition à un allergène. Il a été démontré que l'hyperréactivité des voies aériennes observée dans les cas d'asthme est reliée à l'inflammation de ces mêmes voies aériennes (Pueringer et Hunninghake, 1992). Cependant, on connaît encore très mal les mécanismes par lesquels l'inflammation des voies aériennes peut causer l'hyperréactivité. Il est probable que plusieurs médiateurs, dont les cytokines et les métabolites de l'acide arachidonique, principalement les leucotriènes, libérés par les cellules bronchiques, soient à l'origine de la réaction inflammatoire. Ces médiateurs peuvent activer des cellules inflammatoires mobilisées provoquant la libération additionnelle de médiateurs, ce qui entraîne des manifestations cliniques d'asthme, dont la bronchoconstriction, l'oedème des voies aériennes et l'hyperréactivité (O'Byrne, 1992).

On a identifié plusieurs stimuli comme étant des agents provocateurs et on les a classés soit comme agents physiques (y compris l'exercice et la température), inflammatoires (y compris les allergènes), infectieux (viraux et bactériens), professionnels, alimentaires (y compris les agents de conservation), pharmaceutiques, ou comme polluants atmosphériques (ozone, bioxyde de soufre, bioxyde d'azote et fumée de cigarette) (Black et Armour, 1989).

2.1 ASTHME PROVOQUÉ PAR LES ISOCYANATES

Une cause fréquente de l'asthme dans les pays industrialisés est l'exposition professionnelle à de nombreux produits chimiques. Les isocyanates sont généralement considérés comme la principale cause de l'asthme professionnel, représentant environ 25% de tous les cas dans les pays industrialisés, incluant le cas de la province de Québec (Lagier *et al.*, 1991; Meredith, *et al.*, 1991). Leurs applications industrielles et leur volatilité ont pour conséquence que de nombreux

travailleurs y sont exposés en les inhalant. Parmi les isocyanates, le TDI est probablement le plus important en raison de son fort potentiel sensibilisant. Le TDI est un produit chimique de faible poids moléculaire, hautement réactif, utilisé dans les peintures et comme agent de polymérisation dans la fabrication des mousses de polyuréthanne, des plastiques et des adhésifs (Brown, 1986). On a évalué l'incidence de l'asthme professionnel provoqué par le TDI à approximativement 5-10% des travailleurs en contact avec le TDI (Butcher, *et al.*, 1993). L'asthme professionnel provoqué par le TDI partage plusieurs caractéristiques avec l'asthme influencé par l'immunoglobuline E (IgE), notamment la réponse aux médicaments antiasthmatiques, et certaines particularités pathologiques avec l'asthme non professionnel (Saetta *et al.*, 1992). A ce titre, l'asthme provoqué par le TDI représente un modèle très utile dans l'étude de la pathogenèse de l'asthme en général, y compris l'asthme influencé par l'IgE, l'asthme intrinsèque et l'asthme professionnel.

Des expositions répétées aux vapeurs de TDI chez les travailleurs sont la cause de l'asthme professionnel qui se caractérise par les deux phases, immédiate et tardive, des réactions asthmatiques. L'inflammation des voies aériennes est habituellement observée dans la phase tardive et est associée à la mobilisation de neutrophiles, à un afflux subséquent d'éosinophiles, à une augmentation des niveaux d'albumine dans le liquide de lavage bronchoalvéolaire (BALF)[1] qui suggèrent le développement d'une réaction inflammatoire aiguë des voies aériennes, à une extravasation plasmatique et à la formation d'oedème (Fabbri *et al.*, 1987). Chez ces patients, tant les éosinophiles que les mastocytes semblent avoir subi une dégranulation. On a associé une augmentation du leucotriène (LT) B_4 à la réaction asthmatique tardive; cependant, le LTB_4 étant libéré par les neutrophiles, on ignore s'il est la cause ou la conséquence de l'infiltration des neutrophiles (Zocca *et al.*, 1990). Selon certaines études, l'hyperréactivité qui se manifeste est reliée à la dose (Paggiaro *et al.*, 1986) et entraîne une augmentation de la réactivité aux bronchoconstricteurs non spécifiques comme la méthacholine (Mapp *et al.*, 1988). De plus, une provocation au TDI des individus sensibilisés peut être mortelle (Fabbri *et al.*, 1988).

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[1] N.D.L.T. Pour faciliter la compréhension, compte tenu que ce mémoire est rédigé en anglais, nous utiliserons les abréviations anglaises BAL pour lavage bronchoalvéolaire et BALF pour liquide (fluid) de lavage bronchoalvéolaire.

L'usage prophylactique des corticostéroïdes, comme le béclométhasone et le prednisone, bloque la réaction asthmatique tardive et la réactivité accrue aux bronchoconstricteurs non spécifiques qui y est associée, de même que l'infiltration des neutrophiles et des éosinophiles et l'extravasation de l'albumine, et laisse croire que l'inflammation des voies aériennes est l'élément clé dans l'étude de l'asthme provoqué par le TDI (Mapp *et al.*, 1987; DeMarzo *et al.*, 1988). A l'exception des stéroïdes, la majorité des agents pharmacologiques, y compris le kétotifène (un antagoniste de l'histamine), la théophylline (un inhibiteur de la phosphodiestérase), le vérapamil (un antagoniste des canaux calciques), et le cromoglycate disodique (un stabilisant des mastocytes), qui sont efficaces dans le traitement de l'asthme, se sont avérés inefficaces pour traiter les symptômes de l'asthme provoqué par le TDI (Tossin *et al.*, 1989; Mapp *al.*, 1987). Les mécanismes de l'asthme provoqué par le TDI demeurent inconnus.

2.2 MODÈLES ANIMAUX

L'étude de la physiopathologie de l'asthme, comme de la plupart des maladies, s'appuie sur des modèles animaux. Bien que les modèles animaux permettent d'étudier un grand nombre de paramètres qu'il serait impossible d'étudier chez l'humain sans franchir les limites de l'éthique, les modèles animaux appropriés à la maladie humaine sont difficiles à établir. Une grande variété d'espèces animales ont été utilisées dans les travaux sur l'asthme; le singe, le chien, le mouton, le lapin, le rat, la souris et le cobaye. Parmi toutes ces espèces, le cobaye est certainement l'animal de choix comme modèle de l'asthme humain; il est le plus utilisé, en raison, probablement, de la similitude des réponses physiologiques aux allergènes : réactions asthmatiques immédiate et tardive, hypersécrétion de mucus, infiltration et activation des éosinophiles, et réponses similaires à celles retrouvées chez l'humain en ce qui concerne le traitement avec des bronchoconstricteurs tels que la méthacholine, l'acétylcholine et l'histamine. De plus, ces animaux sont petits et facilement disponibles. Parmi les désavantages, il y a la prédominance possible d'une influence des IgG chez le cobaye alors que l'asthme humain est influencé par les IgE. Cependant, dans quelle mesure les mécanismes influencés par les IgE jouent-ils un rôle dans la pathogenèse de l'asthme ? Bien qu'ils soient d'une importance évidente dans les crises d'asthme de courte durée provoquées par des allergènes chez des sujets atopiques, le rôle des IgE dans la pathogénèse

de la maladie chronique évolutive est beaucoup moins sûr (Corrigan et Kay, 1992). En outre, il n'y a pas d'association entre les IgE et l'asthme professionnel provoqué par les produits chimiques à faible poids moléculaire (Mapp *et al.*, 1994). Par conséquent, bien que cette différence fondamentale suggère que l'on se préoccupe des différences dans les mécanismes de base entre les antigènes à poids moléculaire élevé, comme l'ovalbumine, et les antigènes à faible poids moléculaire comme le TDI, pour les besoins de l'étude présentée ici, le choix du cobaye devient plus pertinent comme modèle de l'asthme provoqué par le TDI chez l'humain.

2.3 LE MODÈLE COBAYE DE L'ASTHME PROVOQUÉ PAR LE TDI

Karol (1983) a étudié la relation entre la concentration de TDI et la production des anticorps spécifiques à TDI, et a découvert une dépendance de concentration inférieure à 1 ppm. De nouvelles études ont démontré une réactivité bronchique en réponse à une provocation par l'histamine après une exposition au TDI et une évidence histopathologique de lésions épithéliales (Cibulas *et al.*, 1986). A partir de ces études les auteurs suggèrent que l'hyperréactivité bronchique peut être reliée aux lésions épithéliales et expriment l'opinion que l'obstruction des voies aériennes n'est vraisemblablement pas reliée à l'hyperréactivité bronchique.

Des études aux microscopes photonique et électronique effectuées par Miller *et al.* (1986) sur les effets de l'exposition au TDI chez le cobaye ont indiqué des changements évidents, comme une inflammation aiguë des voies aériennes après une exposition à 3100 ppb de TDI. Deux heures après la fin de l'exposition, on a observé un remplacement complet de l'épithélium bronchique par des cellules épithéliales stratifiées non kératinisantes. On a également observé un léger oedème des voies aériennes tout comme une augmentation des polymorphonucléaires (PMN) infiltrants, bien que le nombre d'éosinophiles et de mastocytes ne différait en rien de celui des animaux témoins. Vingt-quatre heures après la fin de l'exposition, l'épithélium bronchique et les populations cellulaires infiltrantes étaient inchangés lorsque comparés à ce qu'on avait observé deux heures après l'exposition. Bien que peu ou pas de changement n'ait été observé 72 heures et sept jours après l'exposition, le nombre d'éosinophiles, toutefois, avait augmenté de façon marquée après 72 heures pour atteindre un maximum après sept jours. Trois semaines après l'exposition, l'épithélium avait retrouvé son apparence normale.

Plusieurs études récentes sur les mécanismes de l'hyperréactivité bronchique provoquée par le TDI ont surtout porté sur l'inflammation neurogène: l'inflammation des voies aériennes par suite de la libération de peptides puissants (substance P, neurokinine A et peptide relié au gène de la calcitonine) par les nerfs sensitifs non myélinisés des voies nerveuses non adrénergiques et non cholinergiques (Thompson *et al.*, 1987, Mapp *et al.*, 1993a; Mapp *et al.*, 1993b et Mapp *et al.*, 1993c). On a investigué plus précisément le rôle des tachykinines, protéines de faible poids moléculaire libérées par les nerfs afférents activés, et observées au début de la réaction inflammatoire. Dans certaines études on a eu recours à des antagonistes des récepteurs des tachykinines (substance P spécifique et antagonistes des récepteurs de la tachykinine NK2), à des inhibiteurs des enzymes du métabolisme des tachykinines, et à la déplétion des tachykinines par l'utilisation d'un prétraitement à la capsaicine, un composé extrait de poivrons rouges, qui a démontré qu'elle pouvait désensibiliser certains nerfs sensitifs, dont les nerfs des voies aériennes du cobaye qui libèrent des peptides neuroactifs (substance P, neurokynine A et neuropeptide K). Ces études suggèrent l'importance des tachykinines dans l'hyperréactivité bronchique provoquée par le TDI et que cette hyperréactivité est indépendante des effets connus des tachykinines sur l'oedème des voies aériennes. Ces études combinées portent à croire que l'inflammation neurogène intervient dans la médiation de l'hyperréactivité bronchique après une exposition au TDI, que les prostaglandines peuvent déclencher la libération de tachykinines, et que ce sont les tachykinines qui sont la cause directe des contractions provoquées par le TDI.

3. MATÉRIEL ET MÉTHODES

3.1 MODÈLE ANIMAL

Soixante-six cobayes (*cavia porcellus*) albinos mâles (Hra: (DH) SPF) libres d'anticorps viral, âgés de 26 à 30 jours on été livrés par Charles River Canada Inc. St-Constant (Québec). Chaque cobaye a été individuellement identifié par un tatouage sur l'oreille selon le système AIMS (Animal Identification and Marking System). En raison des problèmes rencontrés lors de la détermination de l'atmosphère de TDI qui ont passablement reculé le point de départ prévu de l'expérience, l'âge des cobayes était approximativement de 11 semaines au début de l'exposition.

Par la suite, les animaux ont été répartis en deux groupes selon une méthode de randomisation stratifiée assistée par ordinateur qui assure l'homogénéité des moyennes et des variances de groupes quant au poids corporel. Les animaux ont été répartis comme suit:

IDENTIFICATION DES ANIMAUX

Jour du sacrifice	Traitement	Identification des animaux selon les évaluations			
		Numéros des animaux	Hyperréactivité	Lavage bronchoalvéolaire	Évaluations histopathologiques
Jour 1 (24h)	Témoin Air	1001-1006	1001-1002	1003-1004	1005-1006
	Exposé au TDI	2001-2016	2001-2005	2006-2010	2011-2016
Jour 7	Témoin Air	1007-1012	1007-1008	1009-1010	1011-1012
	Exposé au TDI	2017-2032	2017-2021	2022-2026	2027-2032
Jour 21	Témoin Air	1013-1018	1013-1014	1015-1016	1017-1018
	Exposé au TDI	2033-2048	2033-2037	2038-2042	2043-2048

Les cobayes du groupe exposé au TDI ont été exposés à une atmosphère de 3 ppm de TDI. Cibulas en 1986 avait déjà démontré que cette concentration était suffisante pour provoquer une hypersensibilité chez le cobaye.

A leur arrivée, les cobayes ont été logés dans des cages individuelles de type "shoe box" munies d'une bouteille d'eau, dans une animalerie dont l'atmosphère et la photopériode étaient contrôlées. Tous les animaux avaient accès *ad libitum* à une nourriture commerciale standard de qualité certifiée, sauf pendant l'exposition et durant le nuit précedant l'autopsie. L'eau du robinet qui avait été préalablement adoucie, purifiée par osmose inversée et stérilisée à l'ultraviolet leur était fournie *ad libitum* (sauf durant l'exposition) dans des bouteilles qui étaient changées tous les deux jours et remplies au besoin. Il n'y avait, ni dans l'eau ni dans la nourriture, de contaminant connu dont on pouvait honnêtement redouter l'effet sur les résultats de cette étude.

3.2 SUBSTANCE À L'ESSAI

Le produit à étudier, le 2,4-toluène diisocyanate (TDI) a été obtenu chez Sigma Chemical Co., St-Louis, MO, USA (numéro de catalogue T-6889). Le produit dont la pureté indiquée était de

99% a été conservé à la température ambiante, à l'abri de la lumière directe et utilisé tel que reçu. Le produit témoin était de l'air conditionné et filtré.

3.3 EXPOSITION PAR INHALATION

3.3.1 Équipement

Dans cette expérience, on a utilisé deux chambres d'inhalation standard faites d'acier inoxydable et de verre où le corps entier de l'animal est exposé. Chaque chambre avait un volume interne d'environ 650 litres. Pendant la période d'exposition, on a placé le groupe complet de cobayes (18 mâles pour le groupe témoin et 48 mâles pour le groupe exposé au TDI) à l'intérieur de la chambre dans des cages à compartiments en grillage d'acier inoxydable dont chaque compartiment mesure approximativement 17cm X 7cm X 10cm.

Au cours de la production des atmosphères de vapeurs d'air ou de TDI, les chambres d'inhalation de même que les systèmes de production associés à chacune des chambres ont été installés dans des hottes de plain-pied séparées et ventilées, qui fonctionnaient sous pression négative pour éviter une contamination possible de l'air de la pièce par des traces de TDI.

3.3.2 Débit d'évacuation des chambres

Le débit d'évacuation de l'air dans les chambres d'inhalation a été fixé à une vitesse d'environ 150 L/min. Ce débit avait été déterminé au cours de tests préliminaires et il avait été jugé suffisant pour maintenir dans la chambre des températures de 20° à 24°C, une humidité relative de 30 à 70% et une concentration d'oxygène d'au moins 19% (avant l'introduction du produit à étudier) avec la charge d'animaux. On a utilisé une pompe à vide à basse pression Sihi pour vider la chambre d'inhalation en respectant le débit requis et acheminer l'air contenant le produit à étudier à travers un système de purification d'air composé d'un filtre grossier de 5 μm et d'un filtre absolu (efficace à 99,97% à 0,3 μm) avant d'évacuer l'air à l'extérieur de l'immeuble.

Le débit d'évacuation était réglé au moyen d'un robinet-vanne situé dans les conduits d'échappement et était contrôlé comme une pression différentielle à travers un rétrécissement (diaphragme de 2cm) dans les conduits d'échappements qui avaient été calibrés contre la circulation d'air. En raison du débit d'évacuation, les chambres d'inhalation fonctionnaient sous pression légèrement négative afin de minimiser les fuites vers l'extérieur.

3.3.3 Mode d'opération des expositions

Chaque groupe d'animaux a été soumis à une exposition du corps entier pendant 5 jours consécutifs à raison de quatre heures par jour, soit à environ 3 ppm de vapeurs de TDI, soit à de l'air conditionné filtré. Les animaux ont été placés dans les chambres d'inhalation et les systèmes de production des vapeurs ont été mis en marche. On a défini le temps zéro comme le moment où 95% de la concentration désirée du produit à étudier se retrouvent dans la chambre.

Pour produire la concentration de l'atmosphère de TDI désirée, on a fait passer une quantité d'air, mesurée et filtrée par un filtre HEPA, à travers une quantité de TDI. Le débit mesuré a été ajusté, selon les exigences, sur la base d'échantillons de l'atmosphère de la chambre prélevés en cours d'exposition. Les vapeurs de TDI ont été diluées avec de l'air filtré avant d'entrer dans la chambre d'inhalation. On a activement produit des vapeurs dans la chambre pendant 240 minutes (les 15 minutes requises pour obtenir une atmosphère équilibrée et les 225 minutes suivantes). Après 240 minutes d'opération continue, on a cessé de produire les vapeurs (le débit dans la chambre était maintenu) et on a laissé la concentration de TDI diminuer graduellement pour atteindre environ 5% de la concentration établie. Les cobayes ont alors été retirés de la chambre d'exposition et ramenés à leurs cages de séjour.

Afin de s'assurer que les cobayes ont tous été exposés aux mêmes concentrations de vapeurs de TDI, on a voulu démontrer avant de les exposer, que l'atmosphère de TDI dans la chambre, au niveau des cages, était homogène en prélevant des échantillons dans les quatre coins et dans le centre de la chambre dans la zone de respiration des cobayes à chacun des deux étages de la chambre d'exposition. Les résultats de ces tests ont indiqué qu'il n'y avait pas de variations notables dans les concentrations de vapeurs de TDI quelle que soit la position des animaux dans la chambre. Cependant, dans le but de neutraliser des variations éventuelles de l'atmosphère, on a déplacé les cobayes chaque jour par rotation des cages dans la chambre afin de s'assurer que tous les animaux recevaient la même dose au cours de la période d'exposition.

3.3.4 Évaluation de l'atmosphère d'exposition

Pour déterminer les concentrations de TDI dans l'atmosphère de la chambre d'inhalation, on a procédé par l'analyse chimique d'échantillons retenus par un solvant à différents temps, et recueillis pendant chaque exposition à un endroit représentatif de la zone de respiration des cobayes. La concentration de TDI dans chaque échantillon a été déterminée par une méthode

colorimétrique (Marcali, 1957), l'absorbance lue au spectrophotomètre à 550 nm et reportée sur une courbe d'étalonnage de TDI tracée contre l'absorbance mesurée.

La courbe d'étalonnage a été préparée en traçant les concentrations connues de TDI de huit solutions standard contre l'absorbance de chacune des solutions lue au spectrophotomètre. Les courbes standards ont été considéré comme acceptables seulement si le coefficient de corrélation (r^2) n'était pas plus bas que 0,990. Les courbes standards produites pendant les 5 jours d'exposition au TDI variaient de 0,992 à 0,998. En plus des solutions standard, deux échantillons pour le contrôle de qualité ont été préparés et lus sur la courbe de façon à vérifier la précision de la courbe d'étalonnage.

On a prélevé des échantillons de l'atmosphère de TDI toutes les heures, après avoir établi une atmosphère équilibrée, en retirant de l'air de la chambre à travers une substance qui absorbait le TDI. On a échantillonné l'atmosphère de TDI de la chambre à une vitesse de 0,90 L/min pendant 4 minutes (volume total de l'échantillon: 3,6 L). Afin d'augmenter la sensibilité de la méthode analytique pour détecter toute contamination de l'atmosphère du témoin air (les expositions à l'air et au TDI étaient effectuées simultanément), l'atmosphère du témoin air a été échantillonné de façon continue pendant toute la durée de l'exposition (un échantillon chaque jour). Cet échantillon a été prélevé de la même façon que les échantillons du groupe exposé au TDI prélevés toutes les heures, sauf que l'échantillonnage de l'atmosphère de la chambre a été fait à une vitesse de 0,94L/min pendant 240 minutes (volume total de l'échantillon: 225 L).

On a évalué la température et l'humidité relative des chambres tous les quarts d'heure durant les expositions des cobayes et elles se situaient respectivement entre 21 et 28°C et entre 21 et 76%.

3.4 LAVAGE BRONCHOALVÉOLAIRE

Le lavage bronchoalvéolaire (BAL) a été utilisé pour prélever des échantillons du liquide qui tapisse l'épithélium de la région bronchoalvéolaire, selon la technique de Sirois *et al.* (1982).

A chacun des temps, on a sacrifié des sous-groupes d'animaux, canulé la trachée et infusé le poumon avec des portions successives de PBS (solution saline tamponnée). Après l'infusion et un léger massage de la cage thoracique, on a aspiré le liquide de lavage bronchoalvéolaire (BALF). Les portions retirées de ces lavages successifs ont ensuite été regroupées et le volume total mesuré. Les suspensions cellulaires ont ensuite été centrifugées et, si nécessaire, le culot débarrassé des globules rouges par choc hypotonique. Après une deuxième centrifugation pour éliminer les débris

cellulaires, une numération cellulaire, une évaluation de viabilité cellulaire et une identification des cellules ont été effectuées.

Une numération cellulaire totale du BALF a été effectuée à l'aide d'un hématimètre. Une formule leucocytaire a été faite sur un frottis (en duplicata) de la suspension cellulaire totale obtenu par "cytospin" et coloré au Wright-Giemsa. La viabilité cellulaire a été évaluée par la technique d'exclusion au bleu trypan.

3.5 TESTS D'HYPERRÉACTIVITÉ

Les tests d'hyperréactivité ont été effectués comme suit: a chacun des temps, on a sacrifié des sous-groupes d'animaux. On a rapidement retiré la trachée et le poumon qu'on a placés dans une solution de Krebs refroidie. Les bronches droite et gauche ont ensuite été délicatement disséquées pour les débarrasser de toute trace de parenchyme et de tissu conjonctif. On a alors isolé la bronche inférieure et on l'a coupée en bandelettes identiques. Cette préparation bronchique a par la suite été installée dans un système de perfusion en cascade. Une fois la préparation équilibrée, on a commencé les injections d'histamine et d'acétylcholine sous forme de bolus.

3.6 ÉVALUATION DES STRUCTURES HISTOLOGIQUES

3.6.1 Préparations histologiques

A chacun des temps expérimentaux, les cobayes appropriés ont été sacrifiés après fixation du poumon par instillation intra-trachéale. Le poumon a alors été prélevé et conservé dans le fixateur. Après avoir taillé le poumon en petites tranches, on a procédé à la déshydratation et à l'infiltration, puis les tissus ont été enrobés dans un mélange plastique, le glycol méthacrylate, par lots, pour assurer un échantillonnage de haute qualité. Les blocs de plastique ont été coupés sur un microtome rotatif muni de couteaux de verre et des coupes individuelles d'une épaisseur de 1,0 μm ont été réalisées et colorées ultérieurement au bleu de toluidine.

3.6.2 Évaluation histomorphométrique

Compte tenu de l'énorme dose de travail que représente l'évaluation histomorphométrique, une première évaluation microscopique de toutes les coupes a été effectuée. Le but de cette première évaluation était de déterminer si l'exposition au TDI avait produit les réactions inflammatoires

anticipées et d'avoir une base sur laquelle on pourrait choisir les structures bronchiques qui serviraient aux évaluations histomorphométriques.

Au cours de ces évaluations, le diamètre des bronchioles où une infiltration avait été observée a été mesuré dans le but de choisir les bronchioles dont le diamètre était le plus approprié à des mesures quantitatives. Bien que les plus grosses bronchioles tendaient à présenter une infiltration plus marquée, leur nombre était par ailleurs limité. Afin de s'assurer qu'un nombre adéquat de bronchioles puissent être étudiées, les plus petites bronchioles présentant de l'infiltration ont été choisies.

Dans l'évaluation quantitative préliminaire, des coupes représentatives d'un animal témoin et d'un animal exposé au TDI de chaque temps de sacrifice ont été examinées. On a choisi les bronchioles dont le diamètre s'approchait de 0,35 mm (la longueur et la largeur du dispositif de mesure) sur chacune des coupes sélectionnées. Comme les bronchioles sont rarement rondes (c.-à-d. en coupe parfaitement transversale), on a défini le diamètre comme la longueur de la plus petite coupe transversale. Dans tous les cas, les bronchioles choisies se situaient entre 0,25 et 0,42 mm (0,7 et 1,2 fois la largeur du dispositif). De plus, on n'a utilisé que les bronchioles dont la longueur était moins de deux fois le diamètre. Pour chaque bronchiole, le dispositif optique était centré dans la sous-muqueuse en périphérie de la bronchiole. On a examiné huit champs pour chaque bronchiole, commençant à "midi" et continuant dans le sens horaire autour de la bronchiole à des intervalles de 45°. Après correction pour le grossissement, chaque champ mesurait 0,097mm X 0,097mm, une taille qui éliminait tout chevauchement des champs. Les mesures de la sous-muqueuse ont été faites à l'aide d'un logiciel commercial d'histomorphométrie.

Le rapport du volume des cellules d'infiltration sur la surface de la sous-muqueuse a été évalué avec la méthode "point counting". Cette méthode consiste à projeter sur l'image une grille et à compter le nombre de points de la grille qui tombent sur les cellules infiltrantes et ceux qui tombent sur la sous-muqueuse. Les résultats sont exprimés en volume cellulaire total par unité de surface. Bien que parmi les cellules comptées on retrouvait des macrophages, des monocytes, des éosinophiles et des neutrophiles et que l'on aurait pu les séparer par type cellulaire, on considère que les données les plus sûres pour démontrer l'inflammation sont celles qui tiennent compte du nombre total de cellules. On a estimé que la subdivision des types cellulaires était inappropriée puisque le nombre de cellules étant plus faible, la précision s'en trouvait par conséquent diminuée.

3.7 ANALYSES STATISTIQUES

Les expériences rapportées dans le présent document devaient originalement faire l'objet de recherches plus approfondies dans lesquelles on aurait traité les cobayes avec des agents pharmacologiques servant à bloquer ou à inhiber les effets de l'exposition au TDI. Cependant, nous n'avons pas poursuivi ces recherches additionnelles étant donné l'ampleur du travail que représente l'évaluation des variations inflammatoires et de l'hyperréactivité bronchique ainsi que des observations histopathologiques et morphométriques. L'analyse statistique des résultats fut jugé nécessaire; par contre, vu le nombre limité d'animaux témoins ($N = 2$ par expérience), le choix judicieux des tests statistiques à appliquer avait une importance considérable. Ainsi, à cause du modèle expérimental, nous avons opté pour l'analyse de variance à deux voies ANOVA. En effet, ce test est idéal pour les comparaisons requises dans une étude comme celle-ci puisqu'il utilise une variance de groupe commune qui augmente sensiblement la fiabilité des résultats d'une telle analyse, et ce, malgré le petit nombre d'animaux témoins. Lorsqu'on compare l'analyse de variance à une voie (ANOVA) et celle à deux voies, la fiabilité (mesurée en degrés de liberté) de l'analyse de variance à deux voies ANOVA est évidente; en effet, l'analyse de variance à une voie ANOVA dispose de 4 à 5 degrés de liberté tandis que l'analyse de variance à deux voies ANOVA en a 17.

Au préalable, la décision d'utiliser l'analyse de variance à deux voies ANOVA est basée sur le fait que la variance entre les animaux témoins et ceux exposés aux vapeurs de TDI après 1, 7 et 21 jours, est homogène. L'homogénité des variances a été établie au moyen du test de Bartlett. L'importance statistique a été démontrée à $P \leq 0,05$ avec l'analyse de variance à deux voies ANOVA et à $P < 0,001$ avec le test de Bartlett.

3.7.1 Données sur le lavage bronchoalvéolaire

Des analyses statistiques furent produites pour démontrer l'effet d'une exposition au TDI sur le nombre total de cellules retrouvées dans le liquide de lavage bronchoalvéolaire (BALF). Compte tenu de l'importance de la variabilité dans le nombre de cellules retrouvées dans le BALF, on a normalisé les comparaisons statistiques du nombre de macrophages, d'éosinophiles et de neutrophiles en pourcentage de cellules totales retrouvées. L'effet signification de l'exposition au TDI sur le nombre total de cellules recueillies dans les voies respiratoires ainsi que la proportion des cellules représentées par les macrophages, les éosinophiles et les neutrophiles, ont été analysée.

Dans tous les cas (nombre total des cellules non transformées, macrophages transformés ARCSIN, pourcentage d'éosinophiles, total logarithmique transformé et pourcentage du comptage de neutrophiles), l'homogénité des variances de groupe a été établie en utilisant le test de Bartlett (non significatif, $P < 0,001$).

3.7.2 Données sur l'hyperréactivité

La signification de l'exposition au TDI sur la réactivité bronchique face à une provocation par un agoniste a été évaluée par comparaison statistique des surfaces sous les courbes dose-réponse (AUC)[2] établie pour les cobayes exposés au TDI et pour les cobayes témoins exposés à l'air. Les surfaces sous la courbe (AUC) ont été calculées individuellement pour chaque animal selon les concentrations d'histamine et d'acétylcholine en appliquant la règle trapézoïde aux données combinées individuelles (réponse contractile, concentration de l'agoniste). Bien que chaque expérience ait été effectuée séparément sur les bronches gauche et droite, comme elles venaient du même animal, on a cru qu'il était plus approprié de les considérer ensemble plutôt que séparément. On a donc créé une troisième variable, appelée Réponse Totale, en additionnant la force contractile exercée par les bronches gauche et droite à chaque concentration de l'agoniste. L'AUC totale a été déterminée pour chaque animal individuellement en appliquant la règle trapézoïde à la réponse contractile combinée des bronches gauche et droite à chaque concentration de l'agoniste.

Pour évaluer statistiquement l'effet de l'exposition au TDI sur la contraction bronchique, on a utilisé l'analyse de variance à deux voies ANOVA de l'AUC TOTALE établie pour l'histamine et l'acétylcholine, respectivement.

Dans tous les cas (les valeurs non transformées AUC de la bronche gauche, de la droite et de l'AUC totale), l'homogénité des variances de groupe a été établie au moyen du test de Bartlett (non significatif $P < 0,001$). Les valeurs non transformées de l'AUC TOTALE ont été utilisées pour determiner la signification statistique.

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[2] N.D.L.T. AUC - abréviation de area under curve (surface sous la courbe).

4. RÉSULTATS

4.1 EXPOSITION PAR INHALATION

Les concentrations moyennes dans la chambre d'inhalation, mesurées pendant les 5 jours où les cobayes ont été exposés était de 2,9 $\pm$ 0,4 ppm. En raison des variations dans la concentration des vapeurs de TDI produites par le système générateur de vapeurs, les concentrations de départ étaient plus variables que prévues lors des études préliminaires. Cependant, pendant les 5 jours qu'a duré l'exposition, les concentrations moyennes de TDI auxquelles ont été exposés les cobayes étaient appropriées à la réponse désirée. La variabilité des concentrations dans les chambres a donc été considérée comme n'ayant aucun impact sur l'interprétation des résultats de cette étude.

L'analyse des échantillons recueillis de la chambre d'inhalation d'air filtré pendant le quatrième jour d'exposition a indiqué la présence de TDI à une concentration de 0,02 ppm. Si l'on considère l'absence de résultats semblables lors des études préliminaires et lors des quatre autres jours d'exposition, l'observation isolée d'une contamination à l'état de traces de l'atmosphère témoin qui a été attribuée à la contamination d'un des réactifs avec le TDI a été jugée non représentative de l'atmosphère à laquelle ont été exposés les animaux témoins.

4.2 LAVAGE BRONCHOALVÉOLAIRE

4.2.1 Nombre total de cellules retrouvées dans le BALF

Le lavage bronchoalvéolaire (BAL) a clairement démontré une infiltration marquée de leucocytes dans les voies aériennes après 5 jours d'exposition au TDI. Lorsque l'on compare avec l'exposition témoin menée simultanément, une augmentation de plus de quatre fois supérieure du nombre total de cellules retrouvées dans le BALF a été observée 1 jour après la fin de l'exposition au TDI. Sept jours après l'exposition, le nombre total de cellules dans le BALF avait diminué à environ deux fois les valeurs des témoins alors qu'après 21 jours, on retrouvait un nombre de cellules approximativement équivalent dans les deux groupes.

4.2.2 Modifications dans les populations cellulaires du BALF

En plus d'une infiltration marquée du nombre total de leucocytes, on a observé dans le BALF des changements évidents dans la représentation proportionnelle des populations cellulaires dans les voies aériennes des cobayes exposés à l'air et au TDI. Des numérations cellulaires ont été

effectuées afin de déterminer le nombre relatif de macrophages, d'éosinophiles et de neutrophiles. Chez les animaux exposés au TDI on a observé que les éosinophiles et les neutrophiles avaient augmenté de 20 à 50 fois respectivement, alors que les macrophages avaient diminué de 2 fois, en comparaison avec les animaux témoins correspondants 1 jour après la fin de l'exposition.

En ce qui concerne les macrophages, une interaction significative entre les temps et entre les groupes a été observée; les comparaisons des données recueillies 1, 7 et 21 jours après l'exposition ont donc été traitées séparément. Les données recueillies chez les animaux témoins sacrifiés 1, 7 et 21 jours après l'exposition n'étaient pas significativement différentes. Cependant, les comparaisons des valeurs obtenues chez les animaux exposés au TDI entre les trois mêmes temps indiquaient que malgré des différences non significatives à 1 et 7 jours après l'exposition, le nombre de macrophages retrouvés après 21 jours était significativement plus élevé qu'après 1 et 7 jours. Lorsqu'on a comparé les données des animaux exposés au TDI avec celles des animaux témoins correspondants, on a noté une réduction statistiquement significative du nombre de macrophages à 1 et 7 jours après l'exposition alors qu'après 21 jours les données étaient statistiquement similaires.

Quant aux éosinophiles, les comparaisons statistiques du nombre de cellules retrouvées dans le BALF ont été effectuées sur des pourcentages obtenus par transformation ARCSIN du nombre total d'éosinophiles. En l'absence d'interaction significative des effets entre les temps et entre les groupes, des comparaisons ont été faites sur les données combinées des trois temps pour chaque groupe d'animaux, témoins et exposés au TDI. Ces analyses ont indiqué que le nombre d'éosinophiles chez les animaux exposés au TDI était significativement plus élevé que chez les témoins mais qu'il n'y avait aucune tendance en termes de signification statistique à mesure que la période de récupération se prolongeait.

Pour ce qui est des neutrophiles, le résultat des analyses est en tous points semblable au résultat obtenu avec les éosinophiles, à la seule différence que les comparaisons statistiques ont été effectuées sur le nombre total de cellules transformé par calcul logarithmique.

4.3 TESTS D'HYPERRÉACTIVITÉ

La provocation des bronches isolées par un agoniste a clairement démontré une hyperréactivité bronchique après cinq jours d'exposition au TDI. Les réponses étaient plus marquées 1 jour après la fin de l'exposition. Exprimées en termes d'AUC TOTALE, les réponses observées 1 jour après l'exposition étaient d'environ 3 à 4 fois celles observées 7 et 21 jours après l'exposition. Les réponses obtenues après 7 et 21 jours étaient similaires mais approximativement 2 à 3 fois celles des valeurs des témoins correspondants.

Les résultats de la provocation par l'acétylcholine ont été similaires à ceux de la provocation par l'histamine. Les comparaisons de l'AUC TOTALE chez les animaux témoins sacrifiés 1, 7 et 21 jours après l'exposition n'ont révélé aucune différence statistiquement significative. Par contre, les mêmes comparaisons chez les animaux exposés au TDI ont démontré que les réactions observées 1 jour après l'exposition étaient significativement plus importantes qu'après 7 ou 21 jours. Il n'y avait cependant aucune différence significative dans les réactions observées entre 7 jours et 21 jours après l'exposition. Comparées aux valeurs de l'AUC TOTALE chez les animaux témoins correspondants, les valeurs de l'AUC TOTALE chez les animaux exposés au TDI se sont révélées significativement plus élevées 1 jour après l'exposition alors qu'elles étaient statistiquement semblables 7 et 21 jours après l'exposition.

L'observation qualitative d'une élévation de la pente des courbes dose-réponse (log) de l'histamine et de l'acétylcholine sur les bronches exposées au TDI illustrait bien l'augmentation de la force de contraction.

4.4 ÉVALUATIONS HISTOLOGIQUES

Une évaluation qualitative de toutes les lames histologiques des tissus prélevés chez les cobayes sacrifiés 1, 7 et 21 jours après la fin des expositions a été effectuée. Lorsqu'on a comparé les coupes des animaux exposés au TDI à celles des animaux témoins, les changements histologiques observés 1 jour après la fin de l'exposition indiquaient clairement une atteinte épithéliale caractérisée par un détachement de l'épithélium, le remplacement de l'épithélium cylindrique cilié, pseudostratifié normal par des cellules épithélioïdes cubiques stratifiées non ciliées, une nette inflammation de la sous-muqueuse et de l'oedème. Sept jours après la fin de l'exposition, le processus de réparation était évident et se caractérisait par l'absence d'un détachement observable de l'épithélium. Bien que l'inflammation de la sous-muqueuse soit encore apparente, elle l'était

à un degré moindre que le premier jour après la fin de l'exposition. De même, l'épithélium n'était pas revenu à son apparence cylindrique ciliée, pseudostratifiée normale. Les préparations histologiques du 21e jour après la fin de l'exposition indiquaient une régénération évidente de l'épithélium et avaient une apparence histologique relativement normale.

L'histomorphométrie a été utilisée dans le but de fournir une évaluation semi-quantitative du ratio du volume des cellules d'infiltration sur la surface de la sous-muqueuse. En se basant sur l'évaluation qualitative de toutes les lames de tous les animaux, un animal témoin et un animal exposé au TDI montrant des lésions représentatives à chaque temps de sacrifice, ont été choisis pour l'évaluation histomorphométrique. Compte tenu des raisons de ce choix, les données obtenues ont été considérées comme une indication de la réponse observée. Cette évaluation a été qualifiée de semi-quantitative parce que les données présentées à chaque temps ont été recueillies à partir d'un seul animal. Bien que l'évaluation de coupes additionnelles aurait permis une analyse statistique des résultats de l'étude histomorphométrique, ce travail supplémentaire fera l'objet de travaux ultérieurs.

5. DISCUSSION

Les travaux exposés ici avaient pour but d'analyser deux caractéristiques majeures et cliniquement applicables à l'asthme : la réaction inflammatoire et l'hyperréactivité bronchique. La réaction inflammatoire a été caractérisée par l'étude du liquide de lavage bronchoalvéolaire et par l'évaluation histomorphométrique de l'infiltration cellulaire dans les voies aériennes et dans la sous-muqueuse bronchique.

Ces études ont été confirmées par des observations histologiques qualitatives. L'hyperréactivité bronchique a été déterminée au moyen des techniques d'organes isolés afin d'évaluer les réponses des bronches à des bronchoconstricteurs non spécifiques.

La concentration visée des vapeurs de TDI dans la chambre d'inhalation était de 3 ppm. Au cours des 5 jours d'exposition, une concentration moyenne globale dans la chambre de 2,9 pm $\pm$ 0,4 a été établie; les concentrations moyennes quotidiennes individuelles variaient entre 2,0 $\pm$ 0,6 ppm et 3,9 $\pm$ 1,9 ppm. Même si les moyennes quotidiennes étaient quelque peu variables et que deux concentrations individuelles dans la chambre étaient énormément éloignées de la concentration visée (0,8 ppm et 9,5 ppm obtenues pendant la première heure du quatrième et du cinquième jour, respectivement), on a estimé que ces deux valeurs n'avaient pas influencé les

résultats des expériences. Des études antérieures (Karol, 1983) ont démontré une dépendance de la concentration dans les réactions immunologiques et physiologiques au TDI après une exposition par inhalation. La courbe dose-réponse indiquait un plateau entre 1 et 10 ppm prouvant ainsi que le seuil de production d'anticorps était d'environ 1 ppm. De la même façon, il a été démontré que le pourcentage de cobayes qui produisaient des anticorps était relié à la concentration, et que les concentrations dépassant 1,0 ppm se traduisaient par la production d'anticorps chez 100% des cobayes exposés.

En raison du seuil de 1 ppm établi par Karol et compte tenu que les symptômes asthmatiques provoqués par le TDI continuent d'être considérés comme étant influencés immunologiquement (Mapp, 1994), nous avons jugé que les concentrations établies dans cette expérience répondaient aux objectifs de notre étude.

L'exposition aux vapeurs de TDI aux concentrations choisies dans ces travaux a apporté des réponses biologiques remarquables. L'estimation des populations cellulaires des voies aériennes par lavage bronchoalvéolaire (BAL) a clairement mis en évidence une infiltration significative de cellules inflammatoires dans les voies aériennes et l'évolution dans le temps de cette réponse. L'augmentation statistiquement significative du début dans le nombre total des cellules d'infiltration retrouvées 1 jour après la fin de l'exposition est revenue au niveau des valeurs témoins trois semaines plus tard. L'infiltration de cellules inflammatoires dans les voies aériennes que suggérait l'augmentation du nombre total de cellules dans le BALF a été corroborée par des études concernant le pourcentage de cellules infiltrantes totales représenté par les macrophages, les éosinophiles et les neutrophiles. Le macrophage, une cellule phagocytaire impliquée dans la défense du poumon, principalement contre les lésions, représente jusqu'à 80-90% des cellules que l'on retrouve dans le BALF. Exprimées en pourcentage des cellules totales dans le BALF, les populations de macrophages étaient significativement inférieures aux valeurs témoins 1 et 7 jours après la fin de l'exposition et étaient revenues au niveau des valeurs témoins trois semaines après l'exposition. Ces données suggéraient que l'augmentation du nombre total de cellules ne dépendait pas de l'infiltration de macrophages mais qu'elle était surtout reliée à l'infiltration de leucocytes polymorphonucléaires (PMN).

Des études similaires sur les éosinophiles et les neutrophiles ont indiqué que, aussi bien le nombre absolu que le pourcentage des cellules totales que représentaient les éosinophiles et les neutrophiles étaient plus élevés chez les cobayes exposés au TDI. L'examen des données reportées sur la courbe indiquait que le pourcentage des cellules totales représenté par les PMN était revenu

à la normale trois semaines après l'exposition. Ces données sur les éosinophiles et les neutrophiles ont été considérées comme une confirmation des données sur les macrophages et on est donc arrivé à la conclusion que les études du BALF démontraient manifestement que l'augmentation du nombre de cellules totales retrouvées dans le BALF était le résultat d'une infiltration de PMN inflammatoires.

A l'exception des travaux récents de Raulf *et al.*, 1995, aucune recherche antérieure sur le BALF n'a été rapportée concernant des cobayes exposés au TDI. Dans les travaux décrits par Raulf *et al.*, les auteurs ont clairement démontré un afflux d'éosinophiles dans les voies aériennes 1 heure après une exposition de 2 heures par jour pendant 5 jours à des concentrations de vapeurs de TDI de 10, 20 ou 30 ppb. Même si Raulf et ses collaborateurs n'ont pas observé d'infiltration significative de neutrophiles, ceci peut être dû à la grande différence entre les concentrations de vapeurs de TDI qu'ils ont utilisées et celles utilisées dans nos études.

L'infiltration d'éosinophiles est davantage corroborée par l'analyse du BALF dans de nombreuses études menées chez le cobaye sensibilisé à l'ovalbumine, un modèle courant de l'asthme humain dans lequel le nombre accru d'éosinophiles dans le BALF est considéré comme un élément caractéristique de la réponse asthmatique tardive (Mauser *et al.*, 1993). Et ce qui est le plus important, c'est que les résultats complets de nos études sur le BALF sont confirmés par des observations semblables dans bon nombre d'études cliniques d'expositions professionnelles aux vapeurs de TDI. Dans ces études, des infiltrations significatives à la fois d'éosinophiles et de neutrophiles ont été observées (Fabbri *et al.*, 1987).

Étant donné la confirmation par les travaux de Raulf et collaborateurs de l'infiltration d'éosinophiles observée dans nos expériences, l'association d'augmentations significatives des taux des médiateurs des lipides dérivés de la 5-lipoxygénase (LTB_4 et LTC_4 /LTD_4/LTE_4)[3] observée par ces auteurs, peut être applicable au modèle caractérisé ici. De plus, la stimulation des cellules du BALF par le calcium ionophore A23187 et par l'acide arachidonique ont provoqué une production accrue du LTB_4. Cette dernière observation a été, de plus, corroborée par une étude menée par Mapp *et al.* (1993a) sur des bronches isolées de cobayes après une exposition au TDI. Ces deux dernières études suggèrent l'importance du produit de la 5-lipoxygénase, le LTB_4 dans la probable cascade qui est manifestement de l'asthme.

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[3] N.D.L.T. Leucotriènes (LT) B_4, C_4, D_4, E_4.

Bien qu'il n'existe pas d'études du rôle du LTB_4 dans la propagation de la réaction asthmatique, les informations auxquelles nous avons accès indiquent que les neutrophiles peuvent jouer un rôle clé dans l'asthme provoqué par le TDI et les autres types d'asthme. A l'heure actuelle, une augmentation du LTB_4 est cliniquement associée à la phase tardive de la réponse asthmatique (Zocca *et al.*, 1990). Cependant, alors que les éosinophiles et les mastocytes libèrent du LTB_4, et que les neutrophiles sont une source importante de cet hypothétique médiateur, on ignore toujours si ce leucotriène joue un rôle dans la mobilisation des neutrophiles ou s'il est la conséquence d'une infiltration de neutrophiles. Le modèle cobaye caractérisé dans l'étude présentée ici offre la perspective d'étudier le rôle des neutrophiles en faisant intervenir la pharmacologie avec des antagonistes spécifiques du LTB_4, ce qui pourra permettre d'élucider le rôle du LTB_4 dérivé des neutrophiles dans cette cascade.

L'évaluation histologique des coupes de bronchioles suivie de l'évaluation histomorphométrique de la mobilisation cellulaire dans la sous-muqueuse bronchique ont complètement confirmé les résultats obtenus dans nos travaux sur le BALF. L'évaluation qualitative préliminaire de toutes les coupes histologiques des poumons des cobayes sacrifiés 1, 7 et 21 jours après la fin de l'exposition indiquait clairement une atteinte épithéliale. Cette atteinte épithéliale était caractérisée par un détachement de l'épithélium, le remplacement de l'épithélium cylindrique cilié, pseudostratifié normal par des cellules épithélioïdes cubiques stratifiées non ciliées, et une nette inflammation de la sous-muqueuse associée à un léger oedème, résultant d'une infiltration de leucocytes polymorphonucléaires. Ces observations étaient particulièrement évidentes 1 jour après la fin de l'exposition au TDI. Le processus de réparation était visiblement amorcé 7 jours après l'exposition et la régénération d'un épithélium d'apparence histologique relativement normale était indéniable 21 jours après l'exposition. Ces constatations concordaient généralement avec les résultats de Miller *et al.* (1986) après 5 jours d'exposition à 3100 ppb de TDI et ceux de Cibulas *et al.* (1986) après 5 jours d'exposition à raison de 4 heures par jour à 3 ppm de TDI.

A la suite de l'évaluation histologique préliminaire, des coupes de poumon considérées représentatives des changements observés à chaque temps de sacrifice ont été choisies pour l'évaluation histomorphométrique. On a qualifié cette évaluation de semi-quantitative parce qu'un seul animal a été analysé à chaque temps. Les éléments obtenus ont corroboré les résultats de l'analyse du BALF. Une importante réaction inflammatoire initiale caractérisée par une infiltration cellulaire a été observée dans la sous-muqueuse bronchique qui, même après 3 semaines, n'était pas complètement revenue à un niveau comparable aux témoins. Un jour après la fin de

l'exposition, le volume des cellules infiltrantes représentait approximativement trois fois la surface de la sous-muqueuse des animaux témoins. Après 7 jours, la surface représentée par les cellules d'infiltration avait diminuée à environ deux fois la surface de la sous-muqueuse des animaux témoins, alors que 3 semaines après la fin de l'exposition, les cellules d'infiltration représentaient environ 1,5 fois les valeurs témoins.

Les données de nos travaux sur le BALF et nos résultats histomorphométriques se sont révélés compatibles avec les résultats du travail morphologique et histomorphométrique rapporté par Miller *et al.* (1986). Nos informations histomorphométriques se sont limitées à une évaluation du pourcentage de la surface de la sous-muqueuse représentée par les cellules infiltrantes (en raison de l'évaluation d'un seul animal à chaque temps de sacrifice, les populations cellulaires individuelles n'ont pas été étudiées). Par contraste, Miller *et al.* (1986) ont évalué le nombre de leucocytes infiltrants (leucocytes polymorphonucléaires, éosinophiles, plasmocytes) séparément dans une étude histomorphométrique 2, 24, 72 heures, 7 et 21 jours après cinq jours d'exposition à raison de 4 heures par jour, à 3100 ppb de TDI. Les résultats ont indiqué que le nombre de PMN atteignait un niveau maximum 2 heures après l'exposition, diminuait progressivement et revenait au niveau des témoins 3 semaines plus tard. Ils ont observé que le nombre d'éosinophiles n'était pas différent des valeurs témoins 2 et 24 heures après l'exposition mais qu'il augmentait significativement, comparé aux valeurs témoins, 72 heures et 7 jours après l'exposition, atteignant un niveau maximum 7 jours après l'exposition. Trois semaines après l'exposition, le nombre d'éosinophiles avait diminué mais demeurait élevé comparativement aux témoins.

Prises dans leur ensemble, ces données ont démontré une différence notable dans le cours du temps d'infiltration des éosinophiles et des neutrophiles dans le poumon du cobaye exposé au TDI. Cette différence représente un contraste potentiellement important avec le tableau clinique et les autres modèles cobayes de l'asthme. Des études récentes chez le cobaye sensibilisé à l'ovalbumine indiquaient que 1 jour après la fin de l'exposition, l'éosinophilie provoquée par l'ovalbumine était beaucoup plus étendue que ce qu'on a observé dans nos études, multipliant par 9 le nombre d'éosinophiles dans le BALF et par 4 ou 5 dans le tissu bronchique intrapulmonaire (Mauser *et al.*, 1993). Des études utilisant les anticorps monoclonaux contre des molécules d'adhésion intercellulaires (ICAM-1) ont démontré une infiltration d'éosinophiles retardée ou inhibée chez le cobaye provoqué par l'ovalbumine (Ihaku *et al.*, 1994). L'expression de la molécule d'adhésion ICAM-1 semble être l'élément clé dans la transmigration endothéliale des leucocytes (Kröegel *et al.*, 1994), et il existe une possibilité que l'exposition au TDI ait un effet soit sur l'expression de

cette molécule ou sur la molécule elle-même suivant son expression, retardant ainsi l'infiltration d'éosinophiles.

Parce que l'infiltration de neutrophiles n'était pas influencée de la même façon, ni dans les travaux de Miller *et al.*, (1986) ni dans les nôtres, la différence dans le temps d'infiltration des neutrophiles et des éosinophiles chez les cobayes exposés au TDI, suggère que les neutrophiles et les éosinophiles puissent être mobilisés par des mécanismes distincts. D'autres investigations utilisant les anticorps monoclonaux spécifiques contre l'ICAM-1 et la E-sélectine, deux molécules d'adhésion soi-disant impliquées dans la transmigration endothéliale des leucocytes, pourraient apporter de l'information additionnelle favorisant l'identification de mécanismes spécifiques de l'infiltration des neutrophiles et, de ce fait, la clarification du rôle de cette cellule dans l'asthme professionnel provoqué par le TDI.

L'évaluation de l'hyperréactivité bronchique utilisant la provocation par un agoniste non spécifique de bandelettes de bronches isolées a clairement démontré une augmentation significative de la réactivité bronchique après une exposition au TDI, réactivité qui est revenue au niveau des témoins à l'intérieur des trois semaines suivant l'exposition. Deux agonistes non spécifiques, l'histamine et l'acétylcholine, ont été utilisés pour réaliser ces expériences. Les résultats obtenus avec chacun de ces agonistes étaient virtuellement identiques: une augmentation statistiquement très significative de la réactivité bronchique observée 1 jour après la fin de l'exposition et un retour à des valeurs statistiquement semblables aux valeurs témoins 7 et 21 jours après l'exposition. L'examen des courbes de la réponse contractile contre la concentration de l'agoniste indiquait une diminution graduelle, 7 et 21 jours après l'exposition, des contractions maximales observées 1 jour après l'exposition. Ces informations ont démontré que les temps choisis pour évaluer la réactivité bronchique étaient appropriés pour caractériser la réponse et ont confirmé les résultats obtenus avec nos autres techniques (lavage bronchoalvéolaire, évaluation histologique, évaluation histomorphométrique semi-quantitative).

Les résultats de nos expériences avec les organes isolés ont appuyé les résultats des expériences physiologiques menées chez l'animal entier par Cibulas *et al.*, (1986). Bien que les données recueillies chez des cobayes exposés au TDI n'étaient pas statistiquement significatives comparées aux valeurs témoins, l'examen de ces données de Cibulas *et al.* (1986) a suggéré que l'absence de signification statistique peut avoir été influencée par la grande variabilité dans les mesures de la SG_{aw} (specific airway conductance) plutôt que par l'absence d'une différence dans la réactivité bronchique entre les animaux exposés au TDI et les animaux témoins 7 jours après la fin de

l'exposition. Bien que la mesure de la PD_{50} (provocative dose, 50%) offre l'avantage d'utiliser comme modèle un animal non anesthésié, il semble que la technique de l'organe isolé employée dans la présente étude soit un indicateur plus sensible de l'hyperréactivité bronchique.

Les résultats des expériences d'hyperréactivité bronchique étaient compatibles avec les constatations histologiques évidentes de lésions épithéliales observées 1, 7 et 21 jours après l'exposition. Ces données concordaient aussi avec la compréhension actuelle du rôle crucial de l'épithélium bronchique dans le maintien du tonus bronchomoteur. Il a été démontré que des cellules épithéliales intactes produisent un facteur relaxant bronchodilatateur, dérivé des cellules épithéliales (EDRF)[4] et récemment identifié comme étant de l'oxyde nitrique (NO) (Moncada *et al.*, 1991). La perte des cellules épithéliales bronchiques se traduisent par une augmentation du tonus des muscles lisses au repos des bronches et par une sensibilité accrue à divers stimuli (Aizawa *et al.*, 1988). Ces réponses ont été confirmées sur des bronches isolées : des études dans lesquelles on a enlevé l'épithélium bronchique des bandelettes de bronches isolées ont démontré une hyperréactivité accrue vraisemblablement due à une perte de NO, et de là une augmentation du tonus bronchomoteur (Prie *et al.*, 1990). La perte de cellules épithéliales en plus de la perte de NO entraînerait une mise à nu des nerfs sensitifs. Il a été suggéré qu'une hyperstimulation ultérieure de ces nerfs cause une bronchoconstriction par les neurones réflexes (Laitinen, 1985).

En conclusion, la caractérisation de la réponse du poumon de cobaye aux vapeurs de TDI décrite dans cette étude fournit des bases solides pour la poursuite de la recherche des causes de l'asthme provoqué par le TDI. Cette caractérisation démontre une excellente corrélation des changements reliés à l'inflammation des voies respiratoires, de l'hyperréactivité bronchique à un agent provocateur et des changements histologiques et morphologiques de l'épithélium bronchique, et ce, dans des conditions établies dans nos laboratoires. Ce modèle peut être utilisé à l'avenir pour étudier les mécanismes de l'asthme provoqué par le TDI par une intervention pharmacologique dans le but d'abolir ou d'inhiber une ou plusieurs réponses observées dans ces études. Le rôle du leucotriène B_4 dans la pathogénèse de l'asthme pourrait être la première cible.

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[4] EDRF: epithelial cell-derived relaxing factor.

THESIS

GENERAL INTRODUCTION

The lungs are the most heavily utilized organs in the body. In addition to being responsible for the uptake of oxygen to maintain metabolism and the elimination of carbon dioxide produced by cellular metabolic processes, the lungs must defend against countless airborne aggressors including pollutants, allergens, viruses, bacteria and microbes. The effects of airborne materials on human health have long been known. As early as the 13th century, concerns over the odor and quantity of coal smoke in the air in London, England reportedly caused the Queen of England to move the royal residence from London to Nottingham. In 1661 public attention was further drawn to the problem of air pollution by John Evelyn who published *"Fumifugium: Or the Inconvenience of the Air and Smoake of London Dissipated"* (sic). By the late 19th century, air pollution problems were recognized in many industrialized countries and the problem continues to worsen. In addition to its obvious potential for environmental damage, the physiological consequences of air pollution came to be acknowledged in periods of still air where large segments of the population began exhibiting signs of respiratory distress some of which developed into chronic respiratory problems similar to asthma or provoked a pre-existing asthmatic condition.

Asthma is an inflammatory disease of the respiratory tract which is characterized by physiologic evidence of airway obstruction (dyspnea, episodic wheezing), airway hyperresponsiveness (an exaggerated degree of airway narrowing which occurs in response to a specific stimulus) and by pathologic evidence of inflammatory changes in the bronchial mucosa (O'Byrne, 1992). This disease is now known to be the only "preventable" disease in which the morbidity and mortality are rising (Page, 1993). In countries where statistics have been compiled, it is estimated that this disease affects 10-15% of the population (Sears, 1990). A variety of stimuli have been identified as provocative agents and have been classified as either physical (including exercise and temperature), inflammatory (including allergens), infective (viral and bacterial), occupational, foods/ preservatives/ drugs, or air pollutants (including O_3, SO_2, NO_2 and cigarette smoke) (Black and Armour, 1989). It has been demonstrated that the airway hyperreactivity observed in asthma is related to inflammation of the airways (Pueringer and Hunninghake, 1992). However, the mechanisms by which airway inflammation may cause airway hyperresponsiveness are still largely unknown. It is likely that mediators released from airway cells initiate the inflammatory response. These mediators may activate recruited inflammatory cells provoking the further release of

additional mediators resulting in the manifestation of clinical asthma including bronchoconstriction, airway edema and hyperresponsiveness (O'Byrne, 1992).

A common cause of asthma in developed countries is occupational exposure to certain chemicals. The most important of these chemicals are widely considered to be the isocyanates. As a consequence of their industrial application and their volatility, human isocyanate exposure via inhalation is widespread. These chemicals are considered to be the principle cause of occupational asthma accounting for approximately 25% of the incidence in the province of Québec and in industrialized countries (Lagier, Cartier and Malo, 1991; Meredith, Taylor and McDonald, 1991). Among the isocyanates, toluene diisocyanate (TDI) is probably the most important due to its high sensitizing potential. TDI is a highly reactive, low molecular weight chemical used in paints and as a polymerizing agent in the manufacture of polyurethane foams, plastics and adhesives (Brown, 1986). Exposure to TDI causes an inflammatory reaction in the airways of animals and man (Gordon *et al.*, 1985; Saetta *et al.*, 1992). At low concentrations, TDI can sensitize workers so that they develop occupational asthma even when re-exposed at levels below the permissible exposure limits (Bernstein, 1982; Mapp *et al.*, 1988). The incidence of TDI-induced occupational asthma has been estimated at approximately 5-10% of TDI workers (Butcher, Mapp and Fabbri, 1993). TDI-induced occupational asthma shares many characteristics of IgE-mediated asthma including the response to anti-asthmatic drugs and the pathological features found in nonoccupational asthma (Saetta *et al.*, 1992). As such, TDI-induced asthma represents a very useful model for the investigation of the pathogenesis of asthma in general including IgE-mediated, intrinsic and occupational asthma. On the basis of similarities to the human response to exposure, the guinea pig provides a good model for TDI-induced occupational asthma. These similarities include respiratory tract inflammation, bronchial hyperreactivity to different bronchoconstrictors and comparable responses to a wide variety of pharmacological agents including steroids and non-steroidal anti-inflammatory drugs (NSAIDs) (Cibulas *et al.*, 1986).

The objective of this research was to characterize the effects of TDI vapor exposure in the guinea pig lung, under the conditions established in our laboratory, through the correlation of inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and morphometric changes in the bronchiolar epithelium.

LITERATURE REVIEW

CHAPTER 1

THE LUNG AS A TARGET ORGAN

1. INTRODUCTION

The lung constitutes the region where the body absorbs the oxygen required to maintain metabolism and eliminates the carbon dioxide produced by cellular metabolic processes. The ability to perform this function lies in the vast amounts of surface area into which the blood and atmospheric air come virtually into contact. In the adult male, the absorptive surface of the lung covers 150 m^2 and is divided amongst more than 300 million alveoli. Of this surface, 80-90% is covered by capillaries for the purpose of gaseous exchange (Weibel, 1973). In a lifetime, the average person will breath over 300 x 10^6 L of air, a volume 5000 times greater than the volumetric intake of food or water (Phalen and Prassard, 1989). These functional requirements have resulted in the evolution of a complex architecture consisting of a hierarchical structure of tubular conduits in which the caliber is progressively reduced in perfect harmony with the laws of fluid mechanics (Weibel and Gil, 1977). The lung has evolved as the organ with the smallest tissue to total organ volume in which a complex fibrous skeleton prevents the collapse of the lung under the normal physical pressures of breathing,

In addition to its obvious potential for attack from airborne toxicants present in inhaled air, the lung is also subject to damage from blood borne substances which have gained entry into the body via routes other than inhalation. Due to its position between the right and left ventricles of the heart, it receives the total cardiac output. Consequently, the lungs can be exposed to various bioactive endogenous compounds and toxic xenobiotic compounds and/or metabolites present in the bloodstream by means of the extensive capillary network (Junod, 1975). Due to the magnitude of the contact between the lungs and toxic substances present in the atmosphere and blood, even at very low levels, these substances can have a marked effect on respiratory function (Smith *et al.*, 1986). In view of its wide potential for toxic injury, the lung is uniquely situated as a target organ for selective damage. The response of the lung to damage can have marked secondary effects on other organs due to the inhibition of normal lung function. In becoming highly differentiated and specialized in their function, cells, including the alveolar epithelial cells, generally tend to lose their

ability to repair or regenerate and are therefore highly susceptible to damage (Frank and Massaro, 1978). Dependant upon the severity of the damage, the resultant recruitment of inflammatory cells and fibrosis may prevent normal lung function and introduce secondary tissue damage due to the failure to maintain cellular metabolism.

In this context, the first part of this chapter attempts to provide an overview of the components of the respiratory tract. This discussion is followed by examples of damage caused by blood borne and air borne toxicants to illustrate the response of lung to injury and to position the discussions of the work undertaken within this project.

2. THE RESPIRATORY TRACT

As many as 40 or more different cell types have been recognized in the airways (Sorokin, 1970; Breeze and Wheeldon, 1977). To study an organ system this complicated, it is useful to subdivide it into different regions. In general, each of these regions are composed of different cell types which contribute to the architecture and function of the various zones. Although compartmentalization schemes vary depending upon the specific goal, the application of the work presented herein is human occupational exposure to an industrial chemical. In this context, the most useful model of the respiratory tract is that presented by the Task Group on Lung Dynamics of the International Commission on Radiological Protection (Morrow *et al.*, 1966) wherein the respiratory tract was divided into three distinct regions: the nasopharyngeal region, the tracheobronchial region and the pulmonary region. Although this model is defined most basically on particulate deposition and clearance phenomena, the divisions simplify the discussions of the respiratory tract. This model is very similar to models presented by the Ad Hoc Working Group to Technical Committee 146 - Air Quality of the International Standards Organization (ISO, 1983) and the Air Sampling Procedures Committee of the American Conference of Governmental Industrial Hygienists (Air Sampling Procedure Committee, ACGIH, 1985) (Phalen and Prassad, 1989).

The first region, the nasopharyngeal region, includes primary defenses against airborne contaminants by means of a mucus coating and ciliated epithelium and is very effective in collecting and eliminating (via the intestinal tract) airborne particulate and other contaminants. The second region, the tracheobronchial region, begins at the larynx and extends down to the terminal

bronchioles. This region is lined with bronchial epithelium and associated submucosal glands and includes the pulmonary arteries, supernumerary arteries and pulmonary veins along with cartilage, smooth muscle and connective tissue. Neither of these regions are involved in gas exchange and are termed the conducting airways. The tracheobronchial region is vulnerable to attack by various compounds which generally generate bronchoconstrictive responses. These responses are discussed in the chapter on the asthmatic response. The third region, the pulmonary region, includes the functional gas exchange sites of the lung: the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. This region is lined with alveolar epithelium (Type I and Type II pneumocytes) together with a vast network of capillary endothelial cells, interstitial cells and macrophages.

The following provides examples of selective lung damage as caused by blood and air borne toxicants:

3. PARAQUAT - SELECTIVE LUNG DAMAGE BY A BLOOD BORNE TOXICANT

Paraquat is a chemical herbicide which has been widely used for more than 20 years. Though low level human exposure can result from inhalation during application, studies of paraquat toxicity were initially performed in response to its use as a means of suicidal agent by oral ingestion (Fletcher, 1974). Although oral ingestion of high single doses results in death due to toxic changes and subsequent failure in the liver, kidney and lung, when more moderate doses are administered, paraquat is selectively toxic to the lung and has been used as a model compound for the study of lung fibrosis. Although the most thorough published studies paraquat toxicity were performed in rats, the pulmonary effects observed in monkeys are similar to those observed in the rat (Murray and Gibson, 1972)

Following intraperitoneal injection of paraquat at a level approximating the oral LD_{50}, evidence was observed within 24 hours of Type I and II pneumocyte damage. Within 2-4 days the alveolar epithelium was completely destroyed. Over time a progressive alveolitis characterized by alveolar edema, hemorrhage and extensive inflammatory cell infiltration into the perivascular areas and interstitium is observed. The majority of treated rats die within a few days of injection. The animals which survive display regenerative changes whereby the more resistant Type II pneumocytes attempt to differentiate into Type I pneumocytes to reline the alveolus to restore the normal alveolar

architecture. The predominant change, however, is the proliferation of fibroblasts within the interstitium of the lung and in the intraalveolar spaces which, along with the remaining edema and alveolar collapse result in death due to anoxia (Vijeyaratnam and Corrin, 1971).

The lung damage is related to the selective uptake of paraquat by Type I and II pneumocytes demonstrated using ^{14}C-labelled material: over a period of approximately 30 hours, plasma paraquat levels remain relatively constant, however lung paraquat levels rise to 6-7 times the plasma levels and are maintained independent of plasma concentration (Smith *et al.*, 1974). This selective uptake is probably a function of structural similarity to a presently unidentified endogenous compounds.

The mechanism by which paraquat damages the lung is generally accepted as being due to redox cycling resulting in the generation of superoxide anion (O_2^{-}) and subsequent generation of hydrogen peroxide (H_2O_2). In the absence of oxygen, paraquat undergoes a single electron reduction to form a stable free radical. This electron is provided by NADPH and is catalyzed by microsomal NADPH-cytochrome C reductase. Under aerobic conditions, the paraquat free radical reacts with molecular oxygen to form (O_2^{-}) and regenerates paraquat. Because O_2^{-} is a stable radical, it is unlikely to be the main toxic species. It will spontaneously react with other superoxide anions to produce H_2O_2, and, if in the presence of a transition metal (such as selenium), it may form highly reactive hydroxyl radical (OH·). In the presence of NADPH and oxygen, this reaction (redox cycling) will continue. This reaction is considered to be the prime reaction in the mechanism of paraquat toxicity.

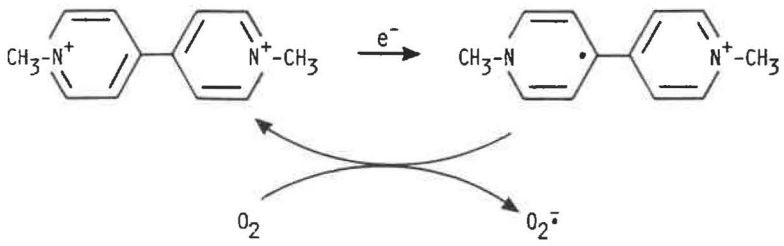


Figure 1 - Paraquat Redox Cycling

These reactive molecules are considered to cause cell damage and death through peroxidation of lipid membranes. An alternative hypothesis is depletion of NADPH. This would result in the inability of the cell to carry out essential physiological and biochemical functions and would result in cell death (Bus and Gibson, 1984).

4. NITROGEN DIOXIDE - SELECTIVE LUNG DAMAGE BY AN AIR BORNE TOXICANT

Nitrogen dioxide (NO_2) is reputed to be the fifth major air pollutant accounting for up to 6% of the total air pollution measured in the USA behind carbon monoxide (52%), sulphur dioxide (18%), hydrocarbons (12%) and particulates (10%). In Los Angeles, the level of NO_2 averages 0.7 ppm. A primary source of NO_2 in industrialized countries is automobile exhaust . This chemical is a deep lung irritant and is capable of producing pulmonary edema if inhaled in sufficient concentrations; respiratory symptoms are evident at concentrations of 5-10 ppm (Timbrell, 1989).

NO_2-induced damage is characterized by terminal bronchiole epithelial cell damage, loss of Clara cell secretory granules and loss of ciliated cells and loss of cilia (Kubota *et al.*, 1987). In cases of extensive exposure, Clara cells can be lost altogether (Côté and Witschi, 1977). Although not involved in gas exchange, the epithelium of the distal bronchiole appears to be the main target tissue of nitrogen dioxide with long term exposure producing the most prominent changes in the terminal bronchiole and adjacent alveoli.

Although the toxicity of nitrogen dioxide is well established, the mechanism of its toxicity is not completely understood. Because NO_2 has an unpaired electron, it can induce autoxidation of organic compounds at concentrations as low as 0.1 ppm (Chao and Jaffe, 1972). Although reduction of intracellular reducing substances such as glutathione also occurs, both *in vitro* and *in vivo* studies indicate that NO_2 initiates autoxidation of unsaturated fatty acids in model systems and in pulmonary lipids of whole animals (Thomas *et al.*, 1968). Furthermore, fatty acid epoxides have been reported in the lungs of NO_2-exposed rats and have been considered indicative of the formation of precursors of lipid peroxides (Sevanian *et al.*, 1979). This evidence was supported by further studies where interaction between NO_2 and pulmonary lipids of surfactant or cell membranes have been demonstrated to lead to initiation of lipid peroxidation (Pryor and Lightsey, 1981). Additionally, lipid peroxidation caused by NO_2 exposure may interfere with receptor-ligand interactions (Patel *et al.*, 1988).

Polyunsaturated fatty acids are particularly sensitive to lipid peroxidation and their presence in cell membranes suggest these membranes are particularly susceptible. The process of lipid peroxidation is initiated by the attack of a free radical on unsaturated lipids and initiates a cascade

of peroxidative reactions which is terminated by the production of lipid breakdown products such as lipid alcohols, aldehydes or smaller fragments such as malondialdehyde. This cascade of reactions ultimately leads to the destruction of the lipid and, in the case of cell membranes, possibly the loss of the structure in which the lipid was located. The effects of lipid peroxidation include adverse effects on the structural integrity altering membrane fluidity, and possibly leading to increased permeability (Timbrell, 1991). Depending upon the membrane affected, this increased permeability can result in leakage of cellular components and potential continued damage.

CHAPTER 2

THE ASTHMATIC RESPONSE

1. INTRODUCTION

Asthma can generally be considered an allergic disease. Historically, it has been considered an immunological disorder in which the binding of immunoglobulin E (IgE) antibodies to mast cells resulted in the release of bronchoconstrictive mediators. This concept has been broadened such that asthma is now considered "a chronic inflammatory disorder of the airways in which many cells play a role including mast cells and eosinophils" (International consensus report on diagnosis and treatment of asthma, 1992). Asthma presents two separate episodes of airway obstruction which are causally unrelated. The early asthmatic response is an episode of bronchoconstriction which typically occurs 10-20 minutes after allergen exposure. The late asthmatic response is a delayed, sustained increase in airway resistance which typically occurs 3-8 or more hours after allergen exposure.

The early response is clearly understood and is a result of immunoglobulin E (IgE) mediated mast cell activation, subsequent degranulation and bronchoconstriction resultant from release of mediators including histamine. This response can be blocked by the use of bronchodilatory β adrenergic receptor agonists and histamine H_1 receptor antagonists. The late response is less understood, and of greater clinical relevance due to the lack of available therapies without significant side effects. Unlike the early response, the late asthmatic response cannot be blocked by H_1 receptor antagonists and is only partially alleviated by β adrenoceptor agonists. Asthma appears to be under the influence of both genetic and environmental factors (Holgate, 1993) and has been illustrated as follows:

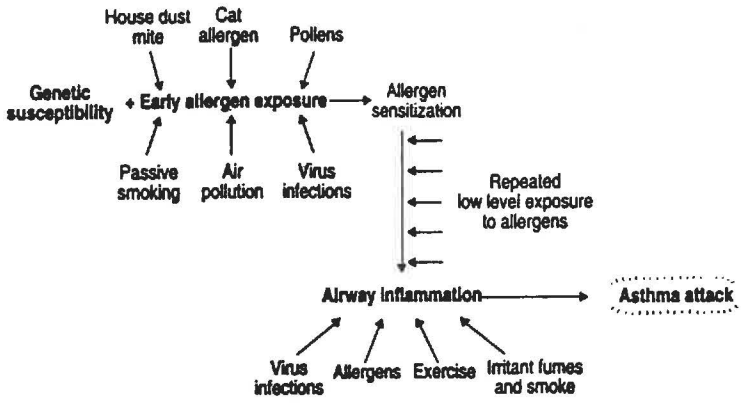


Figure 2 - Potential Genetic and Environmental Factors on Development of Asthma

Clinically, asthma is divided into four grades (I to IV) according to the intensity and frequency of symptoms. Grade I asthma (mild) is characterized by infrequent episodes of acute bronchoconstriction following allergen exposure which are readily alleviated by treatment with β adrenergic receptor agonists. Lung function between acute attacks is normal. In Grade II (moderate), III and IV (severe) asthma, a late response develops which requires long term anti-inflammatory treatment with corticosteroids. In individuals with moderate to severe asthma, lung function between attacks is impaired and their airways are more responsive to irritant substances in the atmosphere (Williams and Shim, 1985). This bronchial hyperreactivity appears to be responsible for the symptoms presented however, cellular inflammatory processes are considered responsible for the development of this hyperreactivity and consequent late phase response.

2. PATHOLOGICAL EFFECTS OF ASTHMA

In addition to the physiological evidence of reversible airway obstruction, bronchial asthma is characterized by pathological evidence of inflammatory changes in the bronchial mucosa. Secondary to the inflammatory changes are airway obstruction caused by smooth muscle contraction and plugging of the airway lumen with mucous. The evidence leading to these conclusions is the following:

1. the severity of asthma correlates well with the number of inflammatory cells present in the airways (Beasley *et al.*, 1989).
2. the extent of airway inflammation correlates with the degree of airway reactivity (Metzger *et al.*, 1987)
3. Asthmatic airway exposure to allergen triggers inflammation (Platts-Mills *et al.*, 1982)
4. only agents which decrease inflammation decrease airway reactivity (Barnes, 1989)

2.1 Bronchial Epithelium

Epithelial damage and shedding are important features of asthma. The bronchial epithelium is widely considered to regulate bronchomotor tone, a conclusion supported by evidence correlating epithelial cell counts with the extent of airway hyperreactivity (Wardlaw *et al.*, 1988). The mechanism by which epithelial damage is thought to contribute to bronchial hyperreactivity is two

fold. The loss of epithelial cells results in the exposure of sensory nerves. Subsequent hyper-stimulation of these nerves causes bronchoconstriction via reflex neurons (Laitinen, 1985). Furthermore, intact epithelial cells have been demonstrated to produce bronchodilatory endothelium-derived relaxant factor (EDRF), recently identified to be nitric oxide (NO) (Moncada *et al.*, 1991). The loss of the bronchial epithelial cells results in an increase in resting airway smooth muscle tone and an increased sensitivity to various stimuli (Aizawa *et al.*, 1988). In addition to these effects, the loss of ciliated bronchial epithelium may also result in a decreased ability to clear mucus secreted into the airways and lead to a further narrowing of the bronchial lumen.

Epithelial cells produce a large number of cytokines and growth factors including interleukin-6 (IL-6), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNFα), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-1). These factors may be released in response to the presence of cytokines, lipid mediators, oxidants, or irritants released into the airways from adjacent cells. There have been indications of increased IL-6 and IL-8 and GM-CSF in the airways of asthmatics (Marini *et al.*, 1992). In addition to cytokines, airway epithelial cells release platelet activating factor (PAF), prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$ and 15-lipoxygenase pathway-derived eicosanoids (Kröegel *et al.*, 1994).

Through the secretion of both pro-inflammatory (ie. GM-CSF) and anti-inflammatory cytokines (ie. transforming growth factor beta [TGFβ]), pulmonary epithelial cells are thought to play a key role in the regulation of pulmonary inflammatory responses by modulation of cellular responses. It has been speculated that the pattern of cytokine release from epithelial cells may determine the nature of the ensuing inflammatory response. Furthermore, lung epithelial cells express intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule considered to be the anchor for various leukocytes including neutrophils, monocytes, lymphocytes and eosinophils during the transmigration of leukocytes into the bronchial mucosa. Evidence suggests that TNF, IL-1 and interferon gamma (IFNγ) contribute to epithelial cell/leukocyte interactions by enhancing ICAM-1 expression on epithelial cells. (Thompson *et al.*, 1995). The importance of this adhesion molecule in the transmigration of leukocytes is supported by studies demonstrating partial blockade of leukocyte infiltration into airways by a monoclonal antibody to ICAM-1 (Kröegel *et al.*, 1994).

2.2 Mast Cells

When compared to non-asthmatics, mast cell numbers can increase 3-5 fold in asthmatics. Compelling evidence indicates IgE-mediated mast cell degranulation results in acute bronchospasm (Beasley *et al.*, 1989). In addition to well known mast cell derived mediators of the early asthmatic response including histamine and serotonin (5-hydroxytryptamine), following *de novo* synthesis mast cells release sulphido-leukotrienes (LTC_4, LTD_4 and LTE_4), PGD_2, and PAF and are believed to synthesize a number of cytokines including IL-3, IL-4, IL-5, IL-6, TNFα and IFNγ (Gordon, Burd and Galli, 1990; Kröegel *et al.*, 1994). Synergistic effects of IL-4 and TNFα released during mast cell degranulation include upregulation of cell adhesion molecules demonstrated to be critical in the potentiation of eosinophil and T-lymphocyte transmigration into the bronchial mucosa (Wasserman, 1994).

Although it is tempting to suggest the mast cell may play a critical role in the initiation of the inflammatory response, evidence which contradicts the potential role of the mast cell exists: treatment with albuterol, one of the most potent inhibitors of mast cell degranulation, did not reduce bronchial reactivity (VanDeGraaf *et al.*, 1991).

2.3 Eosinophils

Eosinophils are probably the most important cells in the pathogenesis of airway inflammation and reactivity. Not only is the presence of increased numbers of these cells within airways characteristic of asthma, the number of cells and quantity of eosinophil products correlates with the severity of the disease (Foresci, 1990). It has become generally accepted that eosinophil infiltration *per se* does not modify airway reactivity, but that the intensity of activation of airway eosinophils may be more relevant to the intensity of bronchial hyperreactivity (Pretolani and Vargaftig, 1994). This is supported by bronchoalveolar lavage (BAL) studies of human asthmatics and animal models in which a higher proportion of hypodense (ie. degranulated) eosinophils were observed in subjects displaying airway hyperreactivity.

Eosinophils are considered to be the mediators of epithelial damage through the release of cytotoxic basic proteins. These proteins are eosinophil granule major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin

(EDN). There is compelling evidence that MBP may be related to the inflammatory changes and tissue damage observed in the bronchial mucosa (Wardlaw *et al.*, 1988).

In addition, eosinophils release a myriad of other factors during the inflammatory process: Human and guinea pig eosinophils contribute to inflammation through *de novo* synthesis and release of lipid mediators such as PAF, LTC_4, LTD_4, 15-HETE, PGD_2, PGF_1, $PGF_{2\alpha}$ and thromboxane A_2 (TxA_2) all of which contribute to tissue inflammation, bronchoconstriction or bronchial hyperreactivity. PGE, an inhibitory prostaglandin, has also been demonstrated and has been shown to down-regulate eosinophil function and may function via a negative feedback signal (Kröegel *et al.*, 1994). Eosinophils synthesize a variety of cytokines including TNFα, GM-CSF, IL-3, IL-5, IL-6 and TGFα and β and appear to secrete both IL-5 and GM-CSF after inhalation challenge with aerosol (Broide, Paine and Firestein, 1992). Eosinophils have been observed to release oxygen metabolites including superoxide anion, hydrogen peroxide and singlet oxygen, all of which are cytotoxic. Finally, there is evidence to indicate that eosinophils may also regulate local inflammation through the release of neuropeptides including vasoactive intestinal protein (VIP) and substance P (Kröegel *et al.*, 1994).

Putative mechanisms involve complicated interactions of several inflammatory cell lines, secreted lipid mediators and cytokines. A general scheme published by Kröegel *et al.* (1994) is illustrated in Figure 3. Briefly, the antigen ① induces the release of IL-4, IL-5 , IL-6 and IFNγ by mast cells which ② regulate proliferation and differentiation of T- and B-lymphocytes ③. B-cells produce IgE which binds to mast cells and recruit basophils ④. T-lymphocytes may differentiate into T-helper (Th_2) cells and release GM-CSF, IL-3, IL-5 and TNFα ⑤. In response to stimulation by GM-CSF, IL-3 and IL-5, circulating eosinophils are primed Ⓐ and endothelial cells present adhesion molecules ⑥. Through the concerted action of mast cell mediators released following IgE binding (which result in the early allergic response) including PAF (the most potent eosinophil chemoattractant known), the expression of eosinophil adhesion molecules may be induced Ⓑ resulting in subsequent eosinophil adhesion Ⓒ and transmigration through the endothelium Ⓓ into the tissue Ⓔ. Continued exposure of the cell to locally secreted cytokines may enhance eosinophil survival and, depending on the nature of the antigen, eosinophil-derived products may either cause destruction of airway epithelium (bronchial asthma) Ⓕ or other local effects (Ⓖ).

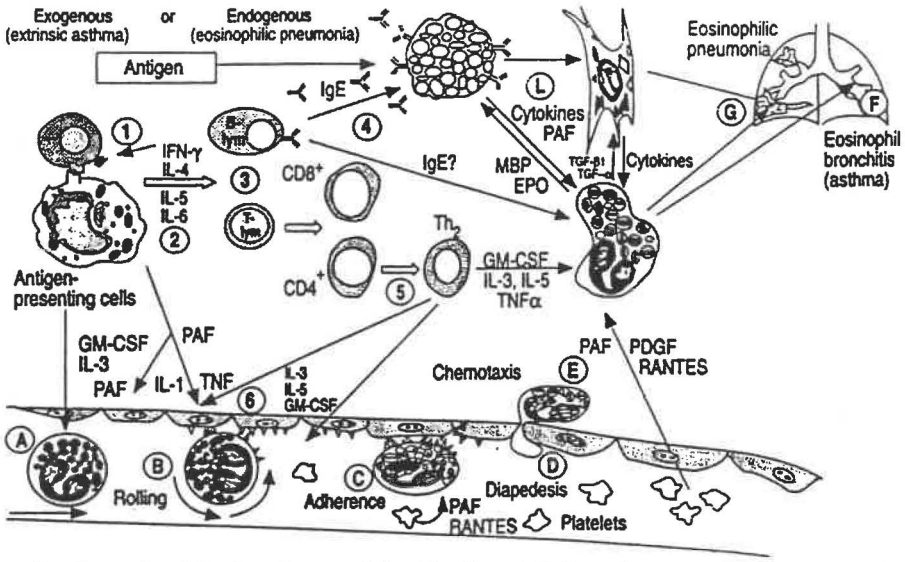


Figure 3 - Putative Mechanisms of the Eosinophil-Dominated Inflammatory Reaction

2.4 T-Lymphocytes

Increasing evidence implicates the T-lymphocytes in the regulation of late-phase reactions associated with asthma. T-lymphocytes are considered effector cells in allergic inflammation and asthma through pathways likely independent of B-lymphocyte regulation and IgE production (Mapp *et al.*, 1994). Whereas most inflammatory mediators recognize passively adsorbed surface immunoglobulins such as IgE, T-lymphocytes appear to be unique in the sense that they can recognize and respond to primary antigens directly. These cells appear to play a pivotal role in initiating and orchestrating ongoing immunologically-driven chronic asthma, particularly in cases where the IgE response is absent or minimal (Corrigan and Kay, 1992). Biopsies of asthmatic airways indicate a tendency for increased numbers of T-cells compared to biopsies from normal subjects early in the asthmatic response (Poston *et al.*, 1992) and a selective increase in Th_1 and Th_2 is observed 48 hours post-challenge in patients previously shown to develop a late response. These data and numerous other studies suggest T-cell recruitment is an initial event in the cascade which results in the late phase reaction (Corrigan and Kay, 1992).

Following allergen recognition, T-lymphocytes release a wide variety of cytokines (lymphokines) in either of two specific patterns: Th_1 cells release IL-2, IFNγ, GM-CSF whereas Th_2 cells release IL-3, IL-4, IL-5 and IL-10. Th_1 cell secretion of IFNγ appears to inhibit Th_2 cell

production; similarly, Th_2 secretion of IL-10 appears to inhibit Th_1 cell production. Th_2 cells are predominant in the airways of asthmatics and have been observed to move rapidly into the airway lumen following allergen exposure.

T-lymphocytes are considered to have significant effects on the recruitment and maintenance of eosinophils: these cells are the source of the two most powerful eosinophil chemoattractants (LCF and IL-2) and Th_2 cells release IL-5, IL-3 and GM-CSF. IL-5 is a cytokine selective for eosinophil growth and differentiation of which, in combination with IL-3 and GM-CSF controls eosinophil production and function and mast cell differentiation (Pretolani and Vargaftig. 1994). Furthermore, IL-4 secretion by Th_2 cells appears to increase expression of VCAM-1 (E-selectin), further facilitating recruitment and activation of eosinophils and lymphocytes.

The ability of these cytokines to prime eosinophils and mast cells via arachidonic acid metabolites suggests a link between the regulatory role of the hematopoietic growth factors and lipid mediators (Pretolani and Vargaftig, 1994). In addition the development of mucosal mast cells is considered to be intimately linked to the production of cytokines by Th_2 cells (Warner and Kröegel, 1994).

2.5 Macrophages

Macrophages represent the single largest cell population recovered in BAL accounting for up to 80-90% of airway cells in the lavage fluid. Macrophages have routinely been divided into two categories: alveolar macrophages, which can be retrieved by BAL, and interstitial macrophages. As it has been recognized that macrophages are found in the airways of the lower respiratory tract, these cells are included within the former category. Study of asthmatic airways indicates that there is no significant difference between the number of macrophages found in the airways of asthmatics when compared with the unafflicted population, however in asthmatics these cells are hypodense (Wardlaw, 1988). Upon allergen exposure, the number of these cells present in asthmatic airways increases markedly due to the infiltration of monocytes into the lung and their subsequent differentiation into macrophages (Metzger *et al.*, 1987). Macrophages release various cytokines upon specific stimulation including IL-1, IL-6, TNFα, TGFβ, fibroblast growth factors (FGF) and PDGF. In addition, they release a wide range of arachidonic acid-derived lipid mediators including

both cyclooxygenase products (TxA_2, PGE_2, PGD_2, and $PGF_{2\alpha}$) and 5-lipoxygenase products (LTB_4, 5-HETE). The predominant mediator released is TxA_2 which is released in ng • mL^{-1} quantities, five times more than LTB_4, which is released in the second largest concentration (Lohmann-Matthes, Steinmüller and Franke-Ullmann, 1994). Several mediators released by macrophages have effects on eosinophils, which, in view of the central role eosinophils play in asthma pathology, are important. These include eosinophil chemoattractants LTB_4, LTC_4 and PAF as well as GM-CSF. In addition, macrophages release other compounds which increase bronchovascular permeability including thromboxanes, TNFα and oxygen reactive species (Brown, Monick and Hunnighake, 1988).

However, due to the lack of specific global macrophage markers, detailed immunohistochemical studies on this cell population in biopsies are few.

2.6 Neutrophils

Although bronchoalveolar neutrophilia is observed before, during and after late (but not early) asthmatic reactions induced by a variety of stimuli, it is not currently known whether neutrophil infiltration is a causative factor or simply a sequela of asthma. The evidence which supports the neutrophil as a causative factor includes inhibition of neutrophil infiltration following corticosteroid pretreatment, and studies on experimentally-induced airway hyperreactivity. Exposure to ozone increases airway hyperreactivity and induces a bronchoalveolar neutrophilia in dogs and man as does exposure to PAF and LTB_4. Furthermore, the effects of PAF and ozone are prevented by neutrophil depletion (O'Byrne *et al.*, 1984). However, the chronic hyperreactivity induced in animal studies is not associated with airway inflammation. Additionally, the airway hyperreactivity induced by ozone develops before the polymorphonuclear leukocyte infiltration of the airway mucosa and is not prevented by corticosteroid pretreatment or leukocyte depletion (Murlas and Roum, 1985). Finally, neutrophil depletion with cyclophosphamide in guinea pigs does not prevent increased airway responsiveness to toluene diisocyanate (TDI) whereas neutrophil depletion with hydroxyurea does, suggesting hydroxyurea may exert a non-specific effect which is responsible for the decreased airway hyperresponsiveness (Thompson *et al.*, 1986).

3. MEDIATORS OF ASTHMA

The characteristic paroxystic bronchoconstriction observed during asthma crises is well known to be a receptor-mediated response to the release of various cell products. Though historical attention has focused on acetylcholine, the neurotransmitter responsible for vagal reflex bronchoconstriction, and histamine released from mast cells during the early phase of the asthmatic response, more recent work has focussed on prostaglandins and leukotrienes, products of phospholipid metabolism, and cytokines, small proteins which are thought to be part of a complex cellular communication network. An overview of the potential involvements of these cell products is presented below.

3.1 Lipid Mediators

Through the action of phospholipase A_2, arachidonic acid is released from membrane phospholipids. Arachidonic acid is subsequently metabolized via two separate pathways into a variety of biologically active compounds including prostaglandins (PGs) via the action of cyclooxygenase or leukotrienes (LTs) via the action of 5-lipoxygenase. The products of arachidonic acid metabolism, the eicosanoids, are highly biologically active. Several are implicated as mediators of asthma.

A schematic representation of the biosynthesis of prostaglandins and leukotrienes in presented in Figure 4.

3.1.1 Cyclooxygenase Products: Prostaglandins and Thromboxane

The various prostaglandins are synthesized in widely different relative amounts by different tissues based upon the relative activity of the various enzymes illustrated in Figure 4. In the lung, prostaglandin synthesis occurs in the endothelial cells (especially prostacyclin synthesis), alveolar macrophages, fibroblasts and type II pneumocytes (Said, 1982). In addition to the respiratory system, prostaglandins and thromboxane have important effects on the cardiovascular and gastrointestinal systems. With respect to the respiratory system, these cell products have both bronchoconstrictive and bronchodilatory activity. For example, tracheal contractions are observed in response to administration of the endoperoxide prostaglandins (PPG_2 and PGH_2) and TxA_2 in the

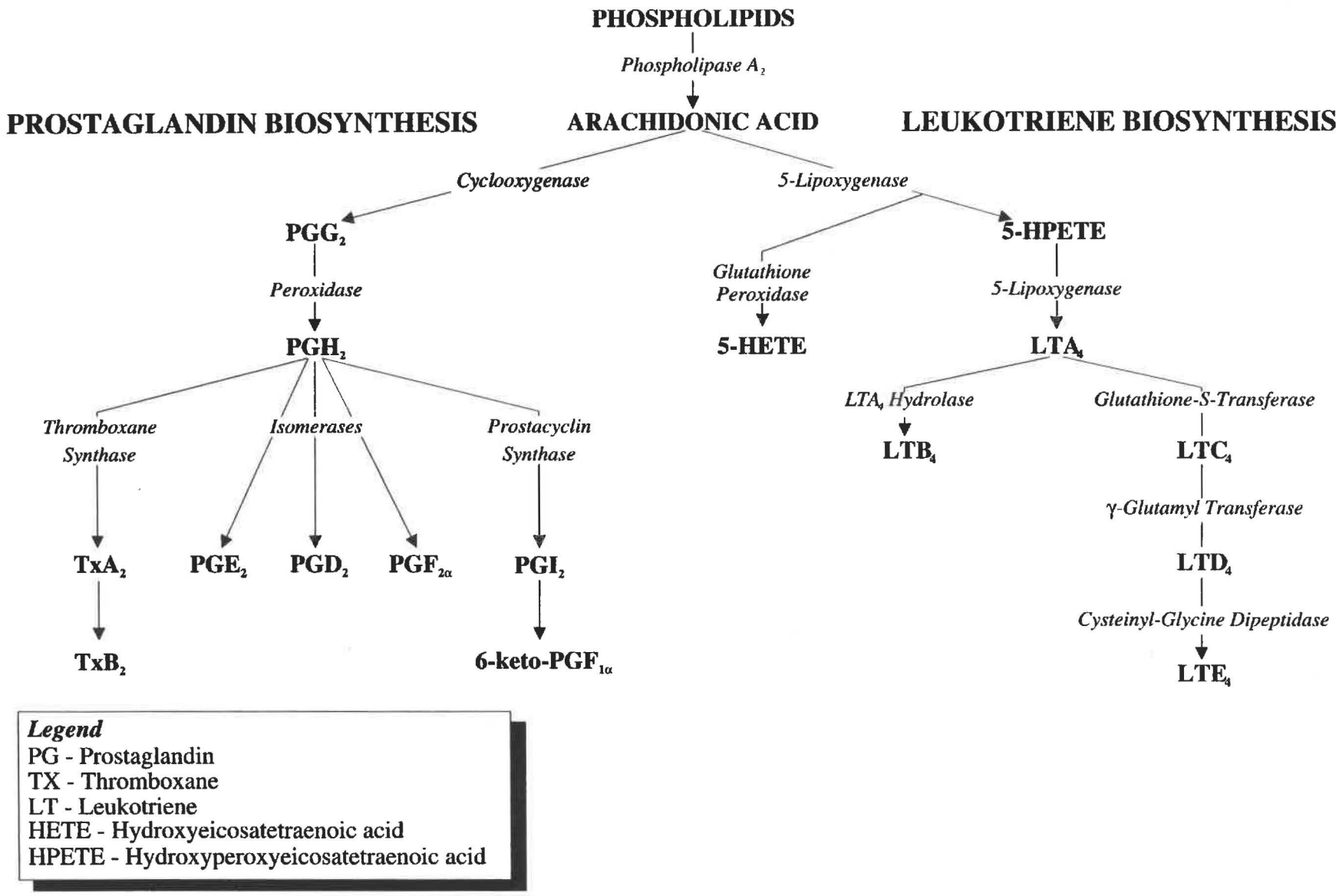


FIGURE 4: ARACHIDONIC ACID METABOLISM - PROSTAGLANDIN AND LEUKOTRIENE BIOSYNTHESIS

guinea pig (Hamberg *et al.* 1976) and by PGD_2 and $PGF_{2\alpha}$ in man (Zijlstra *et al.*, 1987). In man, administration PGD_2 and $PGF_{2\alpha}$ also favorize mucus production (Rich *et al.*, 1984) accentuating the respiratory obstruction. Conversely, PGE_2 and prostacyclin (PGI_2) administration result in a bronchodilatation in man. In addition to direct action on the bronchi, prostaglandins influence lung function through effects on pulmonary circulation. For example, vasoconstriction is observed in response to TxA_2 administration in virtually all species including man.

3.1.2 5-Lipoxygenase Products: Leukotrienes

The leukotrienes are a family of biologically active compounds derived from the metabolism of arachidonic acid via the 5-lipoxygenase pathway. The sulphidopeptide leukotrienes (LTC_4, LTD_4 and LTE_4), originally identified by Borgeat and Samuelsson (1979) as the components of the "slow reacting substance of anaphylaxis" (SRS-A), are considered to be important mediators of asthma. The sulphidopeptide leukotrienes have been estimated to be 100 to 1000 times as potent as histamine as mammalian bronchoconstrictors (Sirois, 1985). In addition, these compounds have been demonstrated to increase mucus production in human and animal respiratory tracts (Piper, 1984). LTB_4 is a chemotactic and chemokinetic agent for inflammatory cells and stimulates the release of TxA_2 from isolated guinea pig parenchymal tissue (Sirois *et al.*, 1982). In addition, LTB_4 induces contractile responses in isolated guinea pig bronchi (Sirois *et al.*, 1987), increases vascular permeability leading to airway edema (Bjork *et al.*, 1982) and is implicated in tissue responses to inflammatory stimuli (Ford-Hutchinson, 1980).

3.2 Cytokines

Cytokines are proteins which are used as an intercellular communications network. These signals function predominantly in a paracrine manner acting on adjacent cells, but may also exert effects in an endocrine (acting on distant cells) or an autocrine (act on cells of the same lineage) fashion. These proteins can be secreted by activated immune and inflammatory cells and there is increasing evidence that these proteins play critical roles in the regulation of the chronic inflammation observed in asthma.

In addition to having overlapping effects, each cytokine has multiple effects which may differ in the presence of other cytokines released from the same cell or the target cell after activation by the cytokine. These properties have made attributing specific effects to any of the greater than 50 cytokines identified to date difficult and make the likelihood of specific cytokine agonists or antagonists unlikely to be of therapeutic benefit for the clinical management of asthma.

Cytokines, specifically IL-4, are thought to play a critical role in inducing and increasing the production of IgE by B cells. The subsequent release of cytokines including IL-1, TNFα and IL-6 may act on epithelial cells and result in the release of a "second wave" of cytokines including GM-CSF, IL-8 and RANTES (regulated on activation, normal T cell expressed and secreted) which serve to amplify the inflammatory response and lead to the infiltration of eosinophils as described in section 2.3. Mast cells directly activated by IgE cross-linking release various cytokines implicated in the differentiation of T-cells to a Th_2 phenotype. Th_2 cell release of cytokines including IL-3, -4 and -5 perpetuate mast cell activation and eosinophilic infiltration. The cytokines released in asthma function in an autocrine fashion perpetuating the inflammatory response (Bittleman and Casalle, 1994).

The specific roles each of these peptides play in the inflammatory reaction and more specifically in asthma have yet to be determined.

4. ANIMAL MODELS OF ASTHMA

Effective study of the pathophysiology of asthma, like most diseases, is based upon animal models. These models have been established because the pathogenic mechanisms of asthma are unknown. Although animal models enable the study of numerous parameters which could not be studied in humans within the bounds of ethics, appropriate animal models of human disease are difficult to establish. To be useful and predictive of the human condition, any putative animal model of human disease must present key characteristics of that disease. The most useful animal models are those which take into account as many of the characteristics of asthma as possible.

A large variety of species have been used for the study of asthma. The differences between the species used as a model and the human must always be identified to ensure that the data obtained is not distorted due to a species-specific etiology. In addition, it is critical that individual studies in

any species be carefully reviewed to ensure the interpretation of data generated is not influenced by abnormal study conditions. It is critical that the specific parameters under study be considered when selecting an animal model: the selection of an animal model for the study of the relationship between genetics and/or environmental conditions on the development of asthma would likely require different criteria be met than would the study of the effect of a specific putative mediator of asthma. Although all animal models present certain similarities to the human condition, it is clear that an isolated similarity does not qualify an animal model as a useful surrogate for the study of human asthma. The selection of an animal model must be based upon the goals of the study and as many similarities to the human condition as possible.

The goal of this research was to characterize the effects of TDI vapor exposure in the guinea pig lung, under the conditions established in our laboratory, through the correlation of inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and morphometric changes in the bronchiolar epithelium. While these features are all considered central to the pathophysiology of asthma, bronchial hyperreactivity has been highlighted by Linssen *et al.* (1991). According to these authors, selection of an animal model for study of bronchial hyperreactivity should take into account the following parameters:

1. ***Causes:*** the model should account for as many causes of bronchial hyperreactivity as possible including airway edema, mucus hypersecretion, recruitment of inflammatory cells into the lung and damage to the respiratory epithelium.
2. ***Anti-asthmatic drug response:*** standard anti-asthmatic agents and especially corticosteroids should be demonstrated to be effective in reduction of bronchial hyperreactivity in the model.
3. ***Bronchoconstrictor response:*** induction of hyperreactivity should result in a general increase in reactivity to a wide variety of non-specific bronchoconstrictors.
4. ***Reproducibility:*** the model must exhibit adequate reproducibility of the uninhibited hyperreactivity over prolonged periods.
5. ***Practicality:*** in order to be effective and allow screening of new therapies the induction of bronchial hyperreactivity must not require complicated and lengthy procedures.

In addition to these criteria, the expense of the test system must be considered. This cost not only includes the initial price of the animals, but also the housing costs associated with the animal species to be used.

The benefits and disadvantages of several species used as animal models of human asthma are discussed below with special attention to the criteria of Linssen *et al.* (1991).

4.1 Monkeys

Monkeys of various strains have been used by a large number of researchers. In addition to the ability to induce sensitization to other antigens, various monkeys exhibit a natural sensitization to *Ascaris suum* (a parasite). In the rhesus monkey, bronchial reactivity appears to be IgE-mediated with *Ascaris* antigen challenge resulting in physiological manifestations of wide spread broncho-constriction. Increased airway responsiveness to non-specific bronchoconstrictors including histamine and $PGF_{2\alpha}$ is also observed and is analogous to the human situation (Krell, 1976). Anti-IgE aerosol challenge of *Ascaris* sensitive monkeys, however, does not produce a consistent response and some monkeys do not react (Patterson and Kelly, 1974). Although studies in *Ascaris* sensitive cynomolgus monkeys indicate a greater proportion of this species are responsive (O'Neill and Goodman, 1981), *Ascaris* sensitive cynomolgus monkeys are difficult to obtain and are very expensive if available.

Recently, Patterson and Harris (1992) described an *Ascaris* sensitive rhesus monkey model in which responses to aerosol challenge are assessed by changes in 5 physiological parameters (respiratory frequency [*f*], pulmonary resistance [R_L], dynamic compliance [C_{dyn}], peak expiratory flow rate [PEFR] and tidal volume [V_T]). In this model, a positive response is considered to be a positive change in four of the five parameters and independent of the magnitude of individual parameter changes. These authors also identified subpopulations of rhesus monkeys based on IgE-mediated allergic responses which were similar to human subpopulations including normal humans, humans with IgE antibody but no asthma, and humans with IgE-triggered asthma.

When coupled with work of other investigators, it becomes apparent that the monkey provides a useful model of human asthma. Other recent studies have focused on the role of the eosinophil in asthma where the numbers of activated eosinophils present in the airways of monkeys correlated

well with the development of bronchial hyperreactivity (Gundel *et al.*, 1990) and that administration of eosinophil major basic protein (MBP) elicited bronchial hyperreactivity (Gundel *et al.*, 1991). All these results are consistent with human asthma.

The predominant characteristics limiting this species as a model for human asthma is the expense of obtaining and maintaining monkeys. This expense largely precludes more than physiological studies such as non-invasive measurements of tidal parameters and specific airway conductance (SG_{aw}). Mechanistic studies which might lead to animal sacrifice are unusual due to the inordinate costs of these animals. As a direct consequence of animal costs, this model becomes limited in the information it can provide and therefore the work performed in this species is limited and the model is relatively under developed when compared to other animal models.

4.2 Dogs

In addition to presenting a natural sensitization to *Ascaris* antigen, sensitization to other antigens can be induced in mongrel dogs. Although the response to *Ascaris* antigen is IgE-mediated, chronic antigen challenge does not increase airway hyperreactivity (Krell and Chakrin, 1976). This model therefore fails to simulate an important clinical feature of asthma. In addition, there is wide variability in responses in the same animal which are not observed in the monkey. This variability and "sudden death syndrome" limit the usefulness of this model (Patterson and Kelly, 1974). Similar models have used ragweed, mixed grass and pollen extract immunization to induce sensitization (Chung, *et al.*, 1985). In this model, dogs are immunized soon after birth and bimonthly thereafter. This regimen resulted in high IgE levels and presented increased R_L within 5 minutes of aerosol challenge. Although a late phase response was not described, inflammatory cells were noted in BAL fluid 6 hours post-challenge in dogs which demonstrated airway hyperreactivity, but not in dogs which did not demonstrate airway hyperreactivity.

A third dog model has been developed by Hirschman *et al.* (1980, 1981a, 1981b) which exploits the natural sensitization to *Ascaris suum* of the Basenji greyhound. In this model, consistent and reproducible airway responses including increased R_L and decreased C_{dyn} are produced following aerosol antigen challenge. This species of dog responds to much lower concentrations of non-specific bronchoconstrictors such as methacholine (10 to 30 times lower) than do mongrel dogs and

therefore offers an advantage over the mongrel dog. This increased responsiveness to non-specific bronchconstrictors is important in the study of the late asthmatic response as the late phase and not the early phase asthmatic response has been associated with subsequent increases in airway reactivity to histamine and methacholine (Larsen *et al.*, 1987). Mechanistic study in the Basenji greyhound has involved an investigation of the relationship between antigen-specific serum IgE, blood leukocyte histamine release and pulmonary responses to *Ascaris* challenge (Peters, Hirschman and Maley, 1982). In this study, pulmonary responses were more closely correlated with the antigen dose required for *in vitro* histamine release from leucocytes than with circulating IgE titer suggesting that antigen challenge is more dependant upon the ease with which mediators are released from pulmonary cells than upon circulating IgE levels.

In spite of several similarities to the human condition, the dog is a doubtful model of human asthma due to the apparent key role played by neutrophils in the pathogenesis of bronchial asthma in this species. While LTB_4 induces non-specific bronchial hyperreactivity in the dog, this hyperreactivity appears to be dependant upon an accumulation of neutrophils and the release of TxA_2 demonstrated by the protective action of cyclooxygenase inhibitors, drugs which are ineffective in the human situation (O'Byrne, 1985). Supportive evidence includes the induction of bronchial hyperreactivity by the TxA_2 mimetic U-46619 (Aizawa and Hirose, 1988). In addition, and similar to the monkey, the expense of obtaining and maintaining dogs and the associated lack of other than physiological studies is a predominant characteristics limiting this species as a model for human asthma.

4.3 Sheep

A less often used model of human asthma characterized by Wanner and Reinhart (1978) is the *Ascaris suum* challenged sheep model. A number of similarities exist between naturally occurring asthmatic and non-asthmatic sheep: there is a differential airway responsiveness between allergic and non-allergic sheep, late responses are associated with recruitment of inflammatory cells (eosinophils and neutrophils) into the airways and prolonged airway hyperresponsiveness, sheep demonstrate acute bronchoconstriction in response to allergen which is readily reversed by β-

agonists and late responses are not reversible with β-agonists but are sensitive to glucocorticoids (Wanner and Reinhart, 1978; Abraham, 1990) .

Despite the wide range of similarities, the predominant features limiting the use of the sheep as a model of human asthma are similar to any large animal species. The sheep is simply not practical for most laboratories due to housing requirements and, when compared with the data that could be generated from rodent studies for the same costs, the sheep is not favored. Other limitations include the limited published data available in this species. As few researchers work with sheep, the characterization of this species as a model of human asthma is minimal when compared with the volumes of data available on the more practical, preferred models such as the guinea pig. Therefore, although the sheep model may be useful in elucidating some of the mechanisms of the pathogenesis of asthma, it is unlikely that this model will be exploited to its fullest.

4.4 Rabbits

With appropriate pretreatment, the rabbit presents many similarities to human asthma and is considered to be a valuable model. Following immunization with *Alternaria tenuis* (a mould), many rabbits will preferentially produce IgE. Responses to aerosol challenge with *Alternaria tenuis* results in early and late phase responses characterized by increased R_L and decreased C_{dyn}, with early phase responses beginning within 15 minutes of challenge and late responses beginning about 100 minutes post-challenge and persisting for a minimum of 6 hours. In addition, antigen challenge of immune rabbits has been demonstrated to lead to several typical features of human asthma including late response mediation by IgE, large airway edema, infiltration of inflammatory cells into the airways and an increase in airway reactivity. Granulocyte depletion has been demonstrated to prevent both the late response and the increased airway reactivity and upon transfusion of a neutrophil-rich cell population into granulocytopenic immune rabbits, both responses were restored (Larsen *et al.*, 1987). Furthermore, from comparison of non-immunized rabbits with rabbits which had been passively immunized with serum from actively immunized rabbits and the observation of no distinguishable difference in airway reactivity to non-specific bronchoconstrictors, it appears that the cellular process occurring during the course of immunization are important for the development of non-specific hyperreactivity in rabbits (Linssen *et al.*, 1991).

The major disadvantage to the rabbit as a model for human asthma is the complex pretreatment required for the immunization. Typically, this process involves antigen injection (either ovalbumin or *Alternaria tenuis*) within 24 hours of birth and again 7, 14, 21, 35, 49, 69 and 77 days after birth (Metzger *et al.*, 1988).

4.5 Rats

Various strains of rats have been used as models for human asthma. Rats release IgE in response to challenge and at least two strains have been demonstrated to display both early and late phase responses following antigen injection (Bellofiore and Martin, 1988; Watanabe and Hayashi, 1990). In studies of Brown-Norway rats, late responders appear to account for *circa* 70% of the populations studied (Eidelman, Bellofiore and Martin, 1988; Bellofiore and Martin, 1988). Because these strains are inbred, the study of genetic influence on asthma, largely precluded by most other species, is possible. In addition, rats demonstrate airway hyperreactivity to nonspecific bronchoconstrictors which persists for several days, but respond poorly to histamine and sulphidopeptide leukotrienes (Watanabe and Hayashi, 1990). However, studies often require the use of adjuvants such as $Al(OH)_3$ or *Bordetella pertussis* to achieve sensitization (Watanabe and Hayashi, 1990). Potential adjuvant effects on the allergic potency or on the mechanism of action make the use of these agents undesirable.

In a comparative study of several rat strains, Watanabe and Hayashi (1990) sensitized Donryu, Lewis, DA and Wistar rats to ovalbumin using an adjuvant (*B. pertusis*) and demonstrated the Donryu rat presented responses most similar to the human situation including biphasic airway responses in which the severity was dependant upon the antigen dose and was correlated with serum IgE levels. The increases in R_L were inhibited with cromolyn sodium (mast cell degranulation inhibitor) and aminophyline (phosphodiesterase inhibitor). In this study, early and late responses were observed to occur within 6 minutes, whereas the early response is observed approximately 5 minutes post-challenge and the late response approximately 120-200 minutes post-challenge in the Brown-Norway rat. As such, the Brown-Norway rat is likely a better model for human asthma (Eidelman, Bellofiore and Martin, 1988).

Unlike the large animal species described above, the rat is ideally suited to most laboratories in that it is small and inexpensive, allowing more detailed mechanistic studies.

4.6 Mice

The mouse is the species of choice for study of the role of cytokines in asthma pathogenesis because immunological tools specific for murine cytokines, growth factors and cell surface receptors are much more well developed than for any other species. These tools have been instrumental in providing fundamental information about putative mechanisms of inflammatory cell recruitment, priming and degranulation (Kröegel *et al.*, 1994). In addition, the small size and low cost of mice allows investigators to conduct large numbers of studies relatively inexpensively. Additional advantages include the major anaphylactic antibody in the mouse is IgE, the demonstration of the development of specific IgE antibody response following antigen inhalation along with increased airway responsiveness (although these changes occur in the absence of airway inflammation) (Renz *et al.*, 1992). In addition, the availability of large numbers of inbred strains allows the study of genetic influence on asthma.

Disadvantages include the fact that the vasculature and not the lung is the primary target of the anaphylactic response making it difficult to extrapolate physiological changes from the mouse to man.

4.7 Guinea Pigs

The guinea pig is certainly the most often used model for testing anti-allergic agents probably because of the marked reactivity of its respiratory system. Many models in this species exist. The review that follows is intended to draw parallels to human asthma. A comprehensive review of studies of guinea pig responses to TDI exposure is presented in Chapter 3.

When compared with man, the general advantages of the guinea pig include a well developed respiratory smooth muscle which responds intensively and rapidly to injection or to inhalation exposure without the need for adjuvants (though some models have use adjuvants), similar physiological responses to challenge including early and late phase responses, inflammatory cell recruitment consistent with asthma and effectiveness of most anti-allergic drugs. Similar to the

mouse, the guinea pig is a small, inexpensive docile animal. Differences include the comparatively important role of histamine and the predominance of IgG rather than IgE as the major anaphylactic antibody (Pretolani and Vargaftig, 1993). In addition, few inbred strains exists limiting the ability to study genetic influence and immunological tools are generally non-existent minimizing the mechanistic study of the basis of asthma in the guinea pig. In the near future, it is likely that specific study of the mechanism of guinea pig responses will be studied with immunological tools as guinea pig-specific monoclonal antibodies are becoming available (Hunt *et al.*, 1993).

Guinea pig models tend to be require either an initial sensitization followed by a challenge with a specific antigen or an exposure to an irritant chemical such as toluene diisocyanate. The small size, ready availability and similarity in physiological responses to allergen when compared with man have resulted in extensive research in this species. In addition to early and late phase asthmatic responses, the guinea pig presents several similarities to the human asthmatic condition. Light and electron microscopic studies have demonstrated mucus hypersecretion and eosinophil infiltration and activation as evidenced by the presence of hypodense eosinophils (Holgate *et al.*, 1988; Ijima *et al.*, 1987). The neutrophil has been demonstrated to have little or no role in guinea pig bronchial hyperreactivity: administration of anti-neutrophil serum inhibited neither the early constriction nor the late phase responses in spite of 90% neutrophil depletion (Hutson *et al.*, 1988). In addition, although marked reactivity is observed, the guinea pig responds to non-specific bronchoconstrictors such as methacholine, acetylcholine and histamine.

A major drawback of the guinea pig as a model for human asthma which is pointed to by several authors is that in contrast to human early asthmatic response mediation by IgE, the guinea pig response appears to be predominantly IgG-mediated limiting mechanistic studies of humoral allergen response (Desquand, 1990). However, because the inflammatory response varies with the choice of protocol, the importance of this difference may be minimized by appropriate experimental design. It has become recognized that large doses of antigen tend to support early and more intense bronchopulmonary hypersensitivity which is primarily mediated by IgG. In contrast, low doses of the sensitizing agent and cyclophosphamide pretreatment has been demonstrated to favor mixed IgG and IgE production (Graziano, *et al.*, 1981). More recently, the importance of the route of administration has been recognized. Although the majority of published studies involve sensitization via injection of antigenic material, sensitization via the respiratory route allows marked

antigen responses in the absence of significant increases in circulating IgG concentrations (Bachelet *et al.*, 1990). In addition, evidence that IgG may confer antigen-specific anaphylactic activity in human skin exists (Parish, 1988).

Furthermore, the extent to which IgE-mediated mechanisms play a role in asthma pathogenesis is uncertain. Insofar as some non-atopic individuals develop the disease whereas not all atopic individuals do, it would appear that IgE-mediated mechanisms are neither necessary nor sufficient for the development of asthma. Although IgE-mediated mechanisms are clearly important in allergen-induced short-term exacerbations of asthma in atopic subjects, the role of IgE in the pathogenesis of the ongoing chronic disease is less certain (Corrigan and Kay, 1992). In addition, there is a lack of association between IgE and occupational asthma developed to low molecular weight chemicals (Mapp *et al.*, 1994). Therefore, although this fundamental difference suggests that consideration be given to differences in basic mechanisms between high molecular weight antigens such as ovalbumin and low molecular weight antigens such as TDI, for the purposes of the study presented herein, the guinea pig becomes more relevant as a model for human TDI-induced asthma.

The importance of specific immunoglobin mediation may not be critical to the elucidation of mechanisms of asthma as demonstrated by a recent study of the role of the eosinophil in the bronchial reactivity in ovalbumin-sensitized and challenged guinea pigs, a model recognized to be mediated by an antibody of the IgG subclass (Pretolani *et al.*, 1994). Similar to the human situation, this study demonstrated that eosinophil activation and not only eosinophil infiltration is required to induce bronchial hyperreactivity. Eosinophil activation was determined by measuring levels of two cationic proteins considered to be markers of activation: eosinophil peroxidase (EPO) and eosinophil-derived major basic protein (MBP). Although only MBP levels were significantly increased in the challenged animals, the authors note that this is consistent with previous work with isolated human eosinophils wherein EPO was selectively generated following anti-IgE but not anti-IgG stimulation (Khaliffe *et al.*, 1986). This difference may result from selective mechanisms responsible for the secretion of the various eosinophil-derived cationic proteins and is consistent with observations of elevated blood MBP but not EPO levels in human asthmatics.

Although the marked reactivity of the respiratory system and the occurrence of respiratory anaphylaxis is sometimes pointed to as a major limitation in this model (Linssen, Wilhelms and Timmerman, 1991), the physiological sequela of antigen challenge are very similar to man. The fact

that certain models require the use of anti-histamine agents to prevent anaphylaxis (ie. ovalbumin sensitization/challenge) may be of concern because histamine has no major indication in human asthma, however, this is not true of all models including the guinea-pig model of TDI-induced asthma model presented in this work.

A number of studies of anti-asthmatic agents have been performed in guinea pigs. Although the effectiveness of β-agonists against early bronchoconstriction has been demonstrated in the guinea pig, there appears to be some effect on late phase reactivity which does not occur in humans (Hutson, Holgate and Church, 1988). Studies of the effects of xanthine (phosphodiesterase inhibitors) and glucocorticoid (phospholipase A_2 inhibitors) administration in toluene diisocyanate challenged guinea pigs have demonstrated similar responses to man: theophylline and enprophylline (xanthines) were observed to reduce the acute inflammatory response, while budenoside (glucocorticoid) was demonstrated to reduce the development of increased airway responsiveness (Erjefält and Persson, 1992).

Similar to the human condition, mechanistic studies in guinea pigs have indicated that the increase in airway responsiveness caused by ozone was not inhibited by treatment with the cyclooxygenase inhibitor indomethacin (Lee and Murlas, 1985). Additional studies indicated this increase was attenuated by various inhibitors or antagonists of metabolites of the 5-lipooxygenase pathway of arachidonic acid metabolism including piriprost (U-60,257), BW755c and FPL-55712 (Murlas and Lee, 1985). Study of the role of cytokines in the ovalbumin challenged guinea pig have indicated that IL-5 plays a key role in the development of bronchial eosinophilia and hyperreactivity with little effect on neutrophilia (Gulbenkian *et al.*, 1992).

When considered together, the evidence presented above and the strong bronchoconstrictive response make the guinea pig an excellent model to study bronchial hyperreactivity. When coupled with the early and late phase responses, eosinophilia and hyperreactive airways, the guinea pig is a useful, relevant model for the study of human asthma.

CHAPTER 3

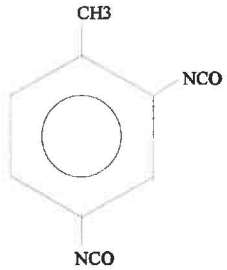
TOLUENE DIISOCYANATE

1. INTRODUCTION

The isocyanates constitute a group of highly reactive, low molecular weight chemicals which are used in a number of industrial and biochemical applications including use in paints and as polymerizing agents in the manufacture of polyurethane foams, plastics and adhesives (Brown, 1986). The major route of human exposure to the isocyanates is by inhalation. As a consequence of their industrial application and their volatility, human isocyanate exposure is widespread. These chemicals are potent sensitizers (NIOSH, 1978) and are considered to be the principle cause of occupational asthma accounting for approximately 25% of the of all cases in industrialized countries as well as Québec (Lagier, Cartier and Malo, 1991; Meredith, Taylor and McDonald, 1991). The incidence of TDI-induced occupational asthma has been estimated at approximately 5-10% of TDI workers (Butcher, Mapp and Fabbri, 1993).

Due to its high sensitizing potential, TDI is perhaps the most important of these compounds. The main use of TDI is in the manufacture of flexible foams in the furniture and automobile industries (Brown, 1986). TDI is a powerful irritant of the mucous membranes of the eyes, gastro-intestinal and respiratory tracts. At low concentrations, TDI can sensitize workers so that they develop occupational asthma even when re-exposed at levels below the permissible exposure limits (Bernstein, 1982; Mapp *et al.*, 1988). Exposure to TDI causes an inflammatory reaction in the airways of animals and man (Gordon *et al.*, 1985; Saetta *et al.*, 1992).

Toluene diisocyanate is an aromatic bifunctional isocyanate and is associated with immunologic sensitization in animal models and some human subjects (Karol, 1985). Due to the presence of the isocyanate groups (-NCO), TDI can react with a variety of functional groups located on many biologically important macromolecules. Potential reaction sites include hydroxyl, sulphydryl and imidazole groups of proteins. Although almost completely insoluble in water, this insolubility may be important with respect to the fate of TDI upon



2,4 Toluene Diisocyanate

FIGURE 5

inhalation. Absorption of TDI through the respiratory tract is likely to occur since high acute toxicity has been reported in animals after inhalation. The most widely available grade of TDI is an 80:20 mixture of the 2,4 and 2,6 isomers (Mapp *et al.*, 1992).

2. TDI-INDUCED ASTHMA: CLINICAL CASE HISTORIES

Repeated exposure to TDI vapors causes an occupational asthma in workers which is characterized by a early and late phase asthmatic reactions. Airway inflammation is commonly observed in the late response and is associated with recruitment of neutrophils, a subsequent influx of eosinophils and an increase of albumin levels in BAL fluid which suggests the development of an acute inflammatory airway reaction, plasma extravasation and edema formation (Fabbri *et al.*, 1987). In these patients, both eosinophils and mast cells appear to be degranulated. Increased levels of LTB_4 has been correlated with the late asthmatic reaction, however because LTB_4 is released by neutrophils, it is not known whether LTB_4 is the cause of neutrophil infiltration or is a result of this process (Zocca *et al.*, 1990). Studies suggest the hyperreactivity which is developed is dose-dependant (Paggiaro *et al.*, 1986) and results in an increase in responsiveness to nonspecific bronchoconstrictors such as methacholine (Mapp *et al.*, 1988). Recent studies of patients with TDI-induced asthma suggest that specific sensitivity to TDI, non-specific airway hyperreactivity and the cellular infiltrate may persist for more than 6 months after cessation of exposure (Saetta *et al.*, 1992). Furthermore, there is documentation of TDI challenge in sensitized individuals being fatal (Fabbri *et al.*, 1988).

Prophylactic use of corticosteroids including beclomethasone and prednisone blocks the late asthmatic response and the associated increases in nonspecific bronchoconstrictor reactivity, the infiltration of neutrophils and eosinophils and the extravasation of albumin and suggests airway inflammation is key to TDI-induced asthma (Mapp *et al.*, 1987; DeMarzo *et al.*, 1988). With the exceptions of steroids, the majority of pharmacological agents which are effective in the treatment of asthma have proved ineffective. Treatment of TDI-induced asthma symptoms with ketotifen (histamine (H_1) antagonist), theophylline (phosphodiesterase inhibitor), verapamil (calcium channel antagonist), and cromolyn sodium (mast cell stabilizer) have all proved ineffective in isocyanate sensitized subjects (Tossin *et al.*, 1989; Mapp *et al.*, 1987).

The mechanism of TDI-induced asthma is unknown. Although the characteristics of TDI suggest an immunological mechanism, whether the humoral response is governed by IgE or IgG has not yet been demonstrated: only a small proportion of individuals with TDI-induced asthma have demonstrated specific IgE antibodies, these antibodies have been observed in unaffected subjects and specific IgG antibodies have been observed (Keskinen *et al.*, 1988). Based on the observation that increased numbers of activated T-lymphocytes (CD25+), activated eosinophils and mast cells have been demonstrated in bronchial biopsies, it is postulated that cell-mediated immunity may play a role in TDI-induced asthma. It is possible that due to their reactive nature, isocyanates are not involved in antibody binding but rather cause a T-cell mediated immune response following reaction with an endogenous protein (Mapp *et al.*, 1994).

3. THE GUINEA PIG MODEL OF TDI-INDUCED ASTHMA

In order to study TDI-induced asthma within the realms of ethics, the guinea pig has been developed as a model. In a study performed by Karol (Karol, 1983), the relationship between TDI concentration and TDI-specific antibodies was investigated. This study indicated that a concentration dependence existed and suggested that above 1 ppm, antibody titers were statistically indistinguishable and were observed in all animals. This study did not address which family of immunoglobulins was responsible for the increased antibody titer.

Further studies in the TDI-exposed guinea pig have been performed to assess bronchial reactivity to nonspecific bronchoconstrictors and investigate the production of IgE-antibodies (Cibulas *et al.*, 1986). After 5 days of 4 hour/day exposure to 3 ppm TDI vapor, serial assessments of bronchial reactivity were performed in unanesthetized guinea pigs following histamine challenge and indicated that airway hyperreactivity occurred in all animals. This hyperreactivity reached a maximum approximately 2 hours after the end of the 5 day exposure period and persisted until 72 hours post exposure completion. Despite the absence of significant hyperreactivity more than 72 hours after the end of exposure completion, airway obstruction (measured as decreased specific airway conductance, SG_{aw}) was observed for 7 days following exposure completion and returned to normal values between 1 and 3 weeks after exposure completion. Investigation of the production of TDI-specific IgE-like antibodies did not reveal IgE-antibody formation, however no investigation

for IgG antibody production were conducted. Histopathological study of the trachea and midright mainstem bronchus was performed at the same intervals following exposure completion as physiological assessments of airway conductance were performed. These studies indicated epithelial damage characterized by replacement of the normal pseudostratified, ciliated columnar epithelium by stratified cuboidal epithelioid cells which were denuded of cilia without mucous droplets. The normal epithelial appearance had not returned three weeks after exposure completion. The cell content of the mucosa indicated highly significant initial elevation in the number of polymorphonuclear leucocytes (PMNs) and cells in mitosis which had returned to normal numbers by 72 and 168 hours post-exposure completion, respectively. Initial marginally low mast cell numbers were significantly increased one and three weeks after exposure completion when compared to controls. From these studies the authors suggested that guinea pig airway hyperreactivity may be related to the airway epithelial damage and suggested that the airway obstruction, although possibly contributory to the decreased specific airway conductance, is likely unrelated to airway hyperreactivity.

Miller *et al.* (1986) studied the effect of TDI exposure on the morphology of the tracheal and bronchial epithelium and Type II pneumocytes. In this experiment, groups of guinea pigs were exposed to 30 or 260 ppb TDI vapor for 5 hours/day for 14 days. A third group of guinea pigs were exposed to 3100 ppb for 4 hours/day for 5 days. Light and electron microscopic study of the trachea, carina, bronchus and Type II pneumocyte was subsequently conducted at intervals following exposure completion. Assessment of the numbers of mast cells, eosinophils, plasma cells and polymorphonuclear cells (PMNs) and intracellular cysts was also performed. While no significant differences from controls was observed in the airway epithelium of the 30 and 260 ppb exposed guinea pigs, obvious change including acute airway inflammation was observed in animals exposed to 3100 ppb.

Two hours after exposure completion, a total replacement of the airway epithelium by stratified non-keratinizing epithelial cells was observed. Minimal airway edema was observed as an increase in infiltrating PMNs, although neither eosinophil nor mast cell numbers differed from controls. Twenty-four hours after exposure completion, the airway epithelia and infiltrating cell populations were unchanged from that observed 2 hours post-exposure. Although little or no change in the airway epithelium was observed 72 hours and 7 days post-exposure, the numbers of eosinophils had

risen markedly by 72 hours post-exposure reaching a peak 7 days post-exposure. Three weeks post-exposure, the epithelium had regained its normal appearance.

Interestingly, cysts were observed within the ciliated cells of control and TDI-exposed guinea pigs was an observation previously noted only in hamsters. These cysts appeared to increase many fold over controls following TDI-exposure.

Other studies of TDI-induced effects in guinea pig include investigations of the role of granulocytes in airway hyperreactivity and edema. Cibulas *et al.* (1988) performed an investigation of the effects of granulocytes (neutrophils and eosinophils) on TDI-induced bronchial hyperreactivity. Using cyclophosphamide (a DNA alkylating agent used in cancer chemotherapy) to induce leukopenia, an assessment of the effects of non-specific bronchoconstrictor challenge following exposure to TDI vapor at *circa* 3 ppm for 10 minutes was performed. In this study, bronchial reactivity was assessed by measuring SG_{aw} following intravenous acetylcholine challenge 2 hours after TDI-exposure. The effect of cyclophosphamide treatment on granulocyte infiltration was assessed by light microscopic examination of tracheal sections obtained from controls and hyperreactive guinea pigs and a manual counting of granulocytes between the epithelial surface and the inner aspect of adjacent cartilaginous tracheal rings. Although cyclophosphamide treatment caused a profound reduction in circulating and airway leukocyte counts, there was no significant effect upon airway hyperreactivity. This study indicated that granulocytes (neutrophils and eosinophils) do not appear to have a role in acute bronchial hyperreactivity following a short, high concentration exposure to TDI vapor.

The role of the granulocyte in airway edema was addressed by Sheppard (Sheppard *et al.*, 1986). In this study, the effect of granulocyte depletion on vascular permeability was studied by assessing the extravasation of a dye (Evan's blue) into the tracheal wall of granulocyte depleted and control TDI-exposed guinea pigs following 1 hour exposure to TDI vapor at a concentration of 2 ppm. Granulocyte-depletion was accomplished with cytotoxic drug pretreatment (vinblastine or hydroxyurea). Although these experiments demonstrated a significant decrease in Evan's blue extravasation into the trachea of granulocyte-depleted guinea pigs, it was uncertain as to whether or not these effects could be attributed solely to the drug induced granulocyte-depletion or another of the widespread effects of cytotoxic drug pretreatment. To support their hypothesis, the authors performed additional studies. Study of the extravasation of Evan's blue following histamine

challenge in granulocyte depleted and control guinea pigs indicated cytotoxic drug pretreatment did not impair the responsiveness of blood vessels in the airways and studies of administration of donor PMNs to guinea pigs previously depleted of granulocytes by hydroxyurea pretreatment indicated control responses were restored. Together, these results suggest the presence of granulocytes is required for TDI-exposure induced airway edema.

Recent studies of the mechanisms of TDI-induced airway hyperresponsiveness have focussed on neurogenic inflammation: airway inflammation as a result of release of potent peptides (including substance P, neurokinin A and calcitonin gene-related peptide) from unmyelinated sensory nerves of the non-adrenergic, non-cholinergic neural pathways of the airways. More specifically, the role of tachykinins, small molecular weight proteins released from activated afferent nerves observed early in the inflammatory response have been investigated. Strategies for study have included the use of tachykinin receptor antagonists (including specific substance P and NK_2-tachykinin receptor antagonists), tachykinin metabolising enzyme inhibitors, and tachykinin depletion using pretreatment with capsaicin, a compound extracted from red peppers which has been demonstrated to desensitize certain sensory nerves including the nerves in guinea pig airways which release neuroactive peptides (including substance P, neurokinin A and neuropeptide K).

In a series of studies investigating the effect of tachykinin depletion and competitive tachykinin inhibition, Thompson *et al.* (1987) exposed guinea pigs to 3 ppm TDI vapor for 1 hour and assessed bronchial hyperreactivity by the effect of acetylcholine aerosol challenge on SG_{aw} in capsaicin and tachykinin receptor antagonist treated and untreated animals. Both capsaicin and tachykinin receptor antagonist pretreatment prevented TDI-induced increases in airway responsiveness to acetylcholine in TDI-treated guinea pigs and did not affect baseline measurements when administered without acetylcholine challenge to TDI-treated controls. Because tachykinins are known to cause airway edema, the possibility that tachykinin depletion inhibited the increase in airway responsiveness by affecting airway vascular permeability was investigated. Studies of capsaicin treatment on TDI-induced tracheal extravasation of Evan's blue dye indicated that airway vascular permeability was not prevented by capsaicin pretreatment. These studies suggest that tachykinins are important in TDI-induced airway hyperresponsiveness and that this effect is independent of the known effects of tachykinins on airway edema.

In several series of studies, the role of tachykinins in this response has been expanded. Using pharmacological techniques, the physiological evidence reported by Thompson *et al.* (1987) has been confirmed (Mapp *et al.*, 1991a): marked decreases in TDI-treated guinea pig bronchial ring contraction was observed in response to acetylcholine challenge following capsaicin pretreatment whereas capsaicin pretreatment did not affect responses to acetylcholine alone. Furthermore, treatment with the tachykinin inhibitor, substance P (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}), reduced the response to acetylcholine challenge following TDI-treatment. These experiments support the earlier studies by Thompson *et al.* (1987) and indicate that tachykinins play an important role in TDI-induced airway hyperresponsiveness. To further investigate the role of tachykinins, the authors conducted studies with the neutral endopeptidase (NEP) inhibitor phosphoramidon (endopeptidases are responsible for tachykinin metabolism). In these studies phosphoramidon treatment was demonstrated to significantly increase the contractile response to acetylcholine challenge following TDI administration while no change in response was observed with phosphoramidon treatment alone.

In combination, these studies suggest that neurogenic inflammation is involved in the mediation of bronchial hyperreactivity following TDI-exposure. Furthermore, the authors suggest that it is possible that TDI might inhibit the metabolic degradation of tachykinins by interfering with endopeptidase activity allowing tachykinins to remain active on bronchial smooth muscle for long periods of time potentially inducing airway hyperresponsiveness.

More recently, Mapp *et al.* (1993a) studied the role of arachidonic acid metabolites in the contractile response of TDI-stimulated isolated guinea pig airways. In this study, guinea pig bronchial rings were prepared and treated with toluene diisocyanate and the production of arachidonic acid metabolites was measured. These assessments were also performed in the presence of the cyclooxygenase inhibitor indomethacin. The arachidonic acid metabolites measured were TxB_2 (the stable metabolite of TxA_2), $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin), PGE_2, and LTB_4, and combined levels of leukotriene C_4, D_4, E_4 and F_4. In contrast to the work of Gordon, Thompson and Sheppard, indomethacin significantly inhibited TDI-induced contraction and the addition of TDI in the presence of indomethacin resulted in unchanged arachidonic acid metabolite levels. These results were consistent with previous experiments by the same authors (Mapp *et al.*, 1991b) and indicated that TDI-induced contractions were associated with increased

levels of the prostanoid mediators $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ but not PGE_2 or the 5-lipoxygenase generated mediators (LTs, TxB_2). These results, when taken in conjunction with studies summarized above and other studies which indicated prostacyclin (PGI_2) activated tachykinin release from capsaicin-sensitive afferent nerves in guinea pig airways (Mapp *et al.*, 1991c), suggests that prostaglandins may trigger tachykinin release and that it is tachykinins which are the direct cause of TDI-induced contractions.

These results contradicted earlier work by Gordon, Thompson and Sheppard (1988). Using physiological evaluations, the potential role of arachidonic acid metabolites in TDI-induced bronchial hyperreactivity were evaluated. In these studies, guinea pigs were exposed to either air or 3 ppm TDI vapor for 1 hour following treatment with a cyclooxygenase inhibitor (indomethacin), a 5-lipoxygenase inhibitor (piriprost) and a combined cyclooxygenase and 5-lipoxygenase inhibitor (BW755c). To compare the effects of drug treatment, dose-response curves to inhaled acetylcholine were constructed and statistical comparisons were made based upon the concentration of the acetylcholine challenge required to increase pulmonary resistance 200% over baseline. The results indicated the airway response to acetylcholine in guinea pigs treated with indomethacin or piriprost were not different from control animals and that treatment with BW755c enhanced airway responsiveness in TDI-exposed animals. In addition to contradicting the results of Mapp *et al.* (1993a), the results obtained with indomethacin and piriprost contradicted studies conducted by Murlas *et al.* wherein study of cyclooxygenase and 5-lipoxygenase inhibitors suggested ozone-induced bronchial hyperreactivity was mediated by a 5-lipoxygenase product, presumably a sulphidopeptide leukotriene (Murlas and Lee, 1985, Lee and Murlas, 1985; see section 4.6). Furthermore, the potentiation of bronchial hyperresponsiveness following BW755c treatment allowed the authors to speculate that TDI may stimulate secretion of a product of either the cyclooxygenase or 5-lipoxygenase pathway which normally attenuates acetylcholine-induced bronchoconstriction. The inhibition of these pathways by BW755c could then explain the increased bronchial responsiveness. The results of these studies must be critically evaluated as arachidonic acid metabolites were not measured and the drug treatments used did not block TDI-induced bronchial hyperresponsiveness. As such, it is uncertain as to whether or not sufficient inhibition of each metabolic pathway occurred.

In a recent clinical study, the authors demonstrated mast cell degranulation was a pivotal event in TDI-induced asthma resulting in the development of airway inflammation (Di Stefano *et al.*, 1993). Because mast cells and other effector cells may be regulated directly and indirectly in their mode of action in the airways by factors such as neuropeptides, and based on other work which indicated that neuropeptides cause secretion of histamine from rat mast cells (Shanahan *et al.*, 1985). Mapp *et al.* (1993b) investigated mast cell involvement in the development of TDI-sensitization using isolated guinea pig bronchial rings and confirmed the clinical observations of Di Stefano *et al.* indicating mast cell activation and degranulation are important events in the allergic response. The role of the mast cell in TDI-induced bronchial hyperreactivity was studied by investigating the ability of antihistamines (combined chlorpheniramine and cimetidine, histamine H_1 and H_2 receptor antagonists, respectively) and compound 48/80 (a chemical which activates tachykinin release from primary afferent nerves and degranulates mast cells) to inhibit TDI-induced contractions. Bronchial rings were prepared and the effects of these drugs were measured as the percentage of the response to acetylcholine challenge following either no pretreatment or TDI exposure. While compound 48/80 treatment caused a concentration-dependant increase in control contractions, degranulation of mast cells by compound 48/80 pretreatment resulted in a significantly reduced response of TDI-sensitized bronchial rings to acetylcholine challenge. Although the antihistamines failed to inhibit acetylcholine-induced responses after TDI exposure, they significantly inhibited responses after histamine and compound 48/80 treatment in non-TDI treated controls. These results indicated that mast cell-mediators other than histamine were involved in the TDI-induced response.

A further series of investigations were focussed on the possible role of tachykinins. These investigations were performed by assessing the magnitude of 48/80-induced contractions following four different pharmacological treatments on capsaicin-sensitive primary afferent nerves: *in vitro* capsaicin desensitization, pretreatment with ruthenium red (a selective functional antagonist of capsaicin-activated transmitter release from bronchial sensory nerves), pretreatment with MEN 10,207 ([Tyr^5,D-$Trp^{6,8,9}$,Arg^{10}]-Neurokinin A, a competitive antagonist of NK_2-tachykinin receptors) and pretreatment with phosphoramidon (a neutral endopeptidase inhibitor).

While compound 48/80-induced contractions were significantly reduced by capsaicin desensitization and were significantly inhibited by NK_2-tachykinin receptor blockade, in contrast to work conducted in TDI-sensitized guinea pig bronchial strips, ruthenium red and phosphoramidon

treatment had no effect. The significant reduction 48/80-induced contractions following capsaicin desensitization indicates that the response to 48/80 is partially dependant upon transmitter-release from primary afferent nerves and partially upon other mechanisms, likely the release of other mediators following mast cell degranulation. Inhibition of 48/80-induced contractions by a selective NK_2-tachykinin receptor antagonist (MEN 10,207) confirms that the response to 48/80 is mediated, at least in part, by tachykinins. The interpretation of the lack of ruthenium red and phosphoramidon effect on 48/80-induced contractions was that 48/80 may activate a different secretory mechanism than does capsaicin.

Because neurogenic inflammation would arise through stimulation of the non-adrenergic, non-cholinergic (NANC) neural pathways present in the airways, and in response to evidence that the loop diuretic bumetanide inhibited both cholinergic and excitatory non-adrenergic, non-cholinergic neurotransmission in guinea pigs, Mapp *et al.* (1993c) studied the effects of this drug in TDI-sensitized guinea pig bronchial rings to determine if neurotransmission blockade could inhibit TDI-induced contraction in response to acetylcholine challenge. However, in doses known to inhibit NANC contraction, bumetanide failed to inhibit the response to TDI.

Although the effects of TDI vapor exposure on the guinea pig lung have been studied by a variety of authors, these studies have not correlated the pharmacological, cytological, morphological and morphometric changes which are characteristic of asthma. Thus, the objective of this research was to characterize the effects of TDI vapor exposure in the guinea pig lung by correlating inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and morphometric effects in the bronchiolar epithelium under the conditions established in our laboratory.

MATERIALS AND METHODS

CHAPTER 4

MATERIALS AND METHODS

1. ANIMALS

Sixty-six male Hartley albino (Hra:(DH)SPF) viral antibody-free (VAF) outbred guinea pigs (*cavia porcellus*) were received from Charles River Canada Inc., St. Constant (Québec). Subsequent to arrival, the guinea pigs were examined to ensure "normal" health status. Each guinea pig was uniquely identified by an ear tattoo using the Animal Identification and Marking Systems (AIMS) procedure.

Upon arrival, the animals were approximately 26-30 days old and weighed between 300 and 350 g. Approximately seven weeks before exposure initiation, all guinea pigs received for this study were weighed and 66 males were assigned to two groups using a stratified computer-based randomization procedure which ensured homogeneity of group means and variances for body weight (this randomization was performed seven weeks prior to animal exposure initiation due to difficulties in validating the method for exposure atmosphere concentration analysis). Animals were randomized as follows:

TABLE NO. 1: Animal Identification

Day of Sacrifice	Treatment	Animal Identification for Evaluations			
		Animal Nos.	Hyperreactivity	Bronchoalveolar Lavage	Histopathological Evaluations
Day 1 (24 h)	Air Control	1001-1006	1001-1002	1003-1004	1005-1006
	TDI-Exposed	2001-2016	2001-2005	2006-2010	2011-2016
Day 7	Air Control	1007-1012	1007-1008	1009-1010	1011-1012
	TDI-Exposed	2017-2032	2017-2021	2022-2026	2027-2032
Day 21	Air Control	1013-1018	1013-1014	1015-1016	1017-1018
	TDI-Exposed	2033-2048	2033-2037	2038-2042	2043-2048

Guinea pigs in the TDI-Exposure group were exposed to an atmosphere of 3 ppm TDI. This concentration was previously shown to be adequate to induce hypersensitivity in the guinea pig (Cibulas *et al.*, 1986). At the onset of exposure, the guinea pigs were approximately 11 weeks old.

The guinea pigs were individually housed in shoe box type bins equipped with a water bottle. All cages were clearly labelled with a color-coded card indicating project, group and animal numbers, sex and dosage level. The animal room environment and photoperiod were controlled (targeted conditions: 12 to 15 air changes per hour, temperature 22 ± 3°C, humidity 50 ± 20%, 12 hours light and 12 hours dark). Actual room temperature and humidity ranged from *circa* 19-27°C and 34-73%, respectively. Deviations from the targeted ranges were isolated and were considered inconsequential.

All guinea pigs had *ad libitum* access to a standard certified commercial laboratory diet (PMI Certified Guinea Pig Chow 5026: PMI Feeds Inc.) except during exposure and prior to necropsy. Maximum allowable concentrations of contaminants in the diet (e.g., heavy metals, aflatoxins, organophosphates, chlorinated hydrocarbons, PCBs) are controlled and routinely analyzed by the manufacturers. Tap water which had been softened, purified by reverse osmosis and sterilized by ultraviolet light was provided *ad libitum* (except during exposure) in water bottles. Bottles were changed every two days and refilled as necessary.

No known contaminants were present in either the food or the water which could reasonably be expected to affect the outcome of this study.

2. TEST ARTICLE

The test article, 2,4-Toluene Diisocyanate (TDI) was obtained from Sigma Chemical, Co., St. Louis, MO, USA (catalog number T-6889). This material was indicated by the supplier to 2,4-toluene diisocyanate at a purity of 99% and was stored at room temperature, out of direct light and was used as supplied. The control article was filtered conditioned air.

3. INHALATION EXPOSURE: ATMOSPHERE GENERATION, CONTAINMENT AND CHARACTERIZATION

3.1 Inhalation Exposure Chamber Design and Operation

Two standard stainless steel and glass whole body inhalation chambers were utilized in this experiment. Each chamber had an internal volume of *circa* 650 L. During exposure, a complete group of guinea pigs (18 Group 1 males and 48 Group 2 males) was housed within the chamber in stainless-steel wire-mesh compartmentalized cages. Each compartment was *circa* 7 inches x 3 inches x 4 inches. The top section of the inhalation chamber had an opening for inlet air into which the test article was introduced. The bottom section of the chamber had a corresponding air extraction port and a drain valve for cleaning the chamber. The chamber was mounted on a transport cart such that the chamber could be easily rotated.

During TDI-vapor of air control atmosphere generation, the inhalation chambers in which the test vapor or air control atmospheres were generated as well as the associated generation system were contained within separate ventilated walk-in fumehoods which were operated at negative pressure to the exposure room to prevent possible contamination of the room air with trace amounts of the test article.

3.2 Chamber Exhaust Flow

The exhaust flow rate through the chambers used to expose the test groups was set at a rate of *circa* 150 L/min. This chamber flow rate was determined in preliminary work to be adequate to maintain a chamber environment of 20 to 24°C, 30 to 70% relative humidity and at least 19% O_2 (prior to test article introduction) with the animal load used in this study.

A Sihi low-pressure vacuum pump was used to exhaust the inhalation chamber at the required flow rates and draw the test article-containing air through an air purifying system consisting of a 5 µm coarse filter and an absolute filter (99.97% efficient at 0.3 µm) before expelling the air from the building.

The exhaust flow was controlled by means of a gate valve located in the exhaust line and was monitored as a differential pressure across a constriction (3/4 inch orifice plate) in the exhaust line. The differential pressure was recorded by a magnehelic gauge. During the prestudy period, a

calibration curve of differential pressure versus mean airflow (measured in triplicate) was prepared. Chamber air flow was recorded 6 times during each exposure: at exposure initiation, concurrently with atmosphere concentration determination after the time required to establish 95% of the target concentration (t_{95}) and hourly during exposure and immediately prior to animals removal from the exposure chamber.

As a result of the exhaust flow, the inhalation chambers were operated under slight negative pressure (*circa* 0.05" H_2O) to minimize outward leakage of the test atmospheres. This pressure difference was established by driving the chamber airflow with the exhaust flow (*circa* 150 L/min) and introducing TDI-vapor at a rate below the exhaust rate. The airflow required to make up the difference between the vapor generator output and the exhaust flow was obtained by drawing air into the chamber from the containment booth. The air supplied to the individual containment booths was HEPA filtered.

In such a dynamic exposure system, the time to reach 95% of the target concentration (t_{95}) is based on the chamber exhaust rate and was calculated as follows:

$$t_{95} = \frac{2.996}{\text{(exhaust rate/chamber volume)}}$$

The constant 2.996 is calculated for 95% of target concentration from the equation:

$$t_x = -\ln[(100 - x)/100]$$

where x represents the percentage of target concentration desired (Cheng and Moss, 1989).

From this equation, the t_{95} was calculated to be 13 minutes (12 minutes, 59 seconds). Similarly, the time required to decay to a concentration that is 5% of the established concentration (t_{05}) was 13 minutes (12 minutes, 59 seconds). For practical reasons, 15 minutes was allowed for the t_{95} and t_{05}.

3.3 Exposure Procedures

Each group of guinea pigs was subjected to a daily four-hour whole body to either *circa* 3 ppm TDI vapor or filtered, conditioned air. The guinea pigs were placed onto the exposure chamber and vapor generation was initiated. Time zero was defined as that point in time at which 95% of the desired concentration of test article in the chamber had been established (after t_{95}).

The desired TDI atmosphere concentration was generated by passing a metered flow of HEPA-filtered air through a frittered gas bubler containing toluene diisocyanate at a rate of 20-30 L/min. The metered flow rate was adjusted as required on the basis of samples of chamber atmosphere obtained during the course of the exposure. The TDI vapor was diluted with HEPA-filtered air prior to entering into the inhalation exposure chamber.

TDI vapor was actively generated for 240 minutes (for the duration required to exceed the t_{95} and a subsequent 225 minutes). Following 240 minutes of continuous operation, vapor generation was stopped (chamber airflow was maintained) and the vapor concentration was allowed to decay to approximately 5% of the established concentration (t_{05}). The guinea pigs were then removed from the chamber and were returned to their home cages.

Although the TDI atmosphere was demonstrated to be homogeneous during the prior to the initiation of exposures, to compensate for any local variations in test atmosphere concentrations, guinea pigs were rotated daily about the exposure chamber to ensure all the animals in the same group received similar doses over the course of exposure.

The filtered, conditioned air control group (Group 1) was restrained and handled in the same manner as the test groups, but was exposed to filtered conditioned air only, using equipment identical to that used for the TDI-exposed group.

3.4 Quantitation of TDI Vapor Concentrations

The concentrations of TDI in the atmospheres of exposed and air control guinea pigs were determined by chemical analysis of time-integrated solvent trap samples collected during each exposure from a representative location at the guinea pig breathing zone. The concentration of TDI in each sample was detemined using a colorimetric method (Marcali, 1957). This method was based on the formation of a reddish-blue color following the rapid hydrolysis of toluene diisocyanate to

toluene diisocyanate diamine, subsequent diazotization of the toluene diisocyanate diamine and final coupling with *N*-1 naphthylethylenediamine to produce a reddish-blue solution. The determination of TDI concentration was made by reading the absorbance of each sample at 550 nm in a spectrophotometer and interpolating the absorbance from a standard curve of TDI concentration plotted against the measured absorbance.

3.4.1 Standard Curve Preparation

The standard curves were constructed by plotting the known TDI concentrations of eight standard solutions against the individual sample absorbance recorded by the spectrophotometer. The analysis required the following solutions. All solutions were made fresh each day of TDI atmosphere sample analysis:

1. Sodium Nitrite (3%): the solution was prepared by dissolving 3.0 g of sodium nitrite and 5.0 g of sodium bromide into about 80 mL of deionized water and adjusting the volume to 100 mL in a volumetric flask;
2. Sulfanic Acid (10%, w/v): 10 g of sulfanic acid was dissolved in *circa* 100 mL deionized water in a 100 mL volumetric flask.
3. *N*-1 Napthylethylenediamine: 50 mg of n (-1-naphthyl)ethylenediamine dihydrochloride was dissolved in *circa* 25 mL deionized water, 1 mL of concentrated hydrochloric acid (11.7*N*) was added and the solution was diluted to 50 mL in a volumetric flask.
4. 1.2*N* Hydrochloric Acid: 51.3 mL of concentrated hydrochloric acid (11.7*N*) was diluted in *circa* 350 mL deionized water and bringing the final volume to 500 mL in a volumetric flask.
5. 8.8 *N* Acetic Acid: in a 1 L volumetric flask, 500 mL of glacial acetic acid (17.6*N*) was mixed with *circa* 400 mL deionized water and subsequently brought to a final volume of 1 L.
6. 0.6*N* Acetic Acid: 17 mL of glacial acetic acid (17.6*N*) was mixed with *circa* 400 mL deionized water in a 500 mL volumetric flask and subsequently brought to a final volume of 500 mL.

The TDI standards were prepared as follows: a stock TDI solution (Solution A) was prepared by weighing 22 mg of pure TDI into a 100 mL volumetric flask containing 60 mL glacial acetic acid (17.6*N*), agitating the solution until the diisocyanate was dissolved and immediately diluting the

solution to 100 mL with deionized water. Due to the reactivity of TDI, Solution A was used within 15 minutes after final dilution. A second solution (Solution B), used to make the TDI standards, was prepared by transferring 1 mL of Solution A into a second 100 mL volumetric flask, adding 6.8 mL of 8.8 *N* acetic acid and diluting the solution to a final volume of 100 mL with deionized water (the final solution was 0.6*N* acetic acid). To a series of eight 25 mL volumetric flasks, increasing volumes of Solution B, decreasing volumes of 0.6*N* acetic acid and 1.2*N* hydrochloric acid were added as follows:

TABLE NO. 2: Standard Curve Preparation for Quantitation of TDI Vapor Concentrations

Standard	1.2*N* Hydrochloric Acid (mL)	0.6N Acetic Acid (mL)	Solution B (mL)	TDI Concentration	
				(ng/mL*)	(ppm**)
A	6.25	12.50	0.00	0 (Blank)	0
B	6.25	11.88	0.625	55	0.28
C	6.25	11.25	1.25	110	0.56
D	6.25	10.00	2.50	220	1.12
E	6.25	8.75	3.75	330	1.68
F	6.25	7.50	5.00	440	2.24
G	6.25	6.25	6.25	550	2.81
H	6.25	0.00	12.50	1100	5.62

* Concentration of TDI in absorbant medium
** Concentration of TDI vapor in chamber atmosphere.

To determine TDI concentration, following preparation each of the standards were processed as follows: to each standard, 0.625 mL of the 3% sodium nitrite solution was added, the mixture shaken and allowed to sit for *circa* 90 seconds. To destroy remaining nitrous acid, 1.25 mL of the 10% sulfanic acid solution was added, the mixture shaken. After allowing the resultant mixture to stand for *circa* 2 minutes, 1.25 mL of the 0.1% *N*-napthylethylenediamine solution was added, the mixture shaken and allowed to stand for the color to develop. After a period of *circa* 5 minutes,

color development was complete and each solution was brought up to a final volume of 25 mL with deionized water.

All samples were analyzed at a wavelength of 550 nm using a 1 cm quartz cell. Prior to each standard analysis, the blank (Standard A) was used to adjust the spectrophotometer to 100% transmittance.

Following the determination of the absorbance of each of the standards, transmittance was plotted against TDI concentration in the final solution using commercial software (Jandel Scientific, TableCurve™ 2D Windows v2.02). The standard curves were considered acceptable if the correlation coefficient (r^2) was no lower than 0.990. The standard curves prepared on the 5 days of TDI exposure had correlation coefficients which ranged from 0.992 to 0.998. To confirm the accuracy of the standard curves, two quality control samples were prepared on each of the 5 days of exposure and their concentrations were determined by interpolation from the standard curve. These QC samples were prepared identically to the standards at TDI concentrations of 88 and 704 ng/mL.

3.4.2 Sample Analysis

Samples of the TDI atmosphere were obtained hourly following establishment of 95% of the target concentration (t_{95}) by drawing chamber air through a sintered glass bubbler containing 15 mL of an absorber medium at a rate of 0.90 L/min for 4 minutes (total sample volume of 3.6 L). To increase the sensitivity of the analytical method in detecting any contamination of the air control atmosphere (air control and TDI-exposures were conducted concurrently), the air control atmosphere was sampled continuously throughout exposure (one sample was obtained from the air control atmosphere each day). This sample was obtained similarly to the hourly samples collected from the TDI-exposed group except that the chamber atmosphere was sampled at a rate of 0.94 L/min for 240 minutes (total sample volume of 225 L).

The absorber medium was prepared in a 1000 mL volumetric flask by adding 35 mL of concentrated hydrochloric acid (11.7*N*) to 22 mL glacial acetic acid (17.6*N*) and diluting the solution to 1000 mL with deionized water (final concentration 0.4*N* hydrochloric and acetic acids). Similarly these samples were processed and analyzed as described above for the TDI standards. The

concentration of TDI in each sample was interpolated from the standard curve based upon the transmittance at 550 nm.

3.5 Atmosphere Homogeneity Assessment

Prior to guinea pig exposure, the homogeneity of TDI vapor concentration within the inhalation chamber was assessed. Chamber atmosphere samples were collected and analyzed as detailed above from five locations on each of the two levels on which guinea pigs were exposed. These samples were obtained towards the four corners and in the center at the guinea pig breathing zone. The coefficient of variation calculated from all samples collected represented the total variation in TDI-vapor concentration with chamber position and time.

These analyses were performed twice and indicated mean concentration of 2.3 and 1.8 ppm with corresponding standard error of the means (s.e.m.) of 0.15 and 0.12 ppm, respectively. These results indicated that there were no remarkable variations in TDI vapor concentration with chamber position. To compensate for any local variations in test atmosphere concentrations, guinea pigs were rotated daily about the exposure chamber to ensure all guinea pigs in the same group received similar doses over the course of exposure.

3.6 Monitoring of Chamber Environmental Conditions

Chamber temperature and relative humidity were assessed at the guinea pig exposure level using an Abbeon-Lufft dial gauge. The chamber temperature and relative humidity were recorded quarter-hourly and ranged from 21 to 28°C and from 21 to 76% during guinea pig exposures, respectively.

4. BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage was used to sample the epithelial lining fluid of the bronchoalveolar region of the lung using the technique of Sirois *et al.* (1982).

On each occasion, subsets of animals were sacrificed by exsanguination following pentobarbitone injection (40 mg • kg^{-1}). Immediately following exsanguination, the trachea of each animal was cannulated with a catheter joined by a three way stopcock attached to two 60 ml

syringes. Using the first syringe, a total of 60 mL of phosphate-buffered saline (PBS, pH 7.4 at 37°C) was infused into the lungs in 5 mL aliquots. Following infusion and gentle massage of the thorax, the bronchoalveolar lavage fluid (BALF) was aspirated using the second syringe. The fluid recovered from these successive lavages was pooled and the volume recovered was measured.

The resulting cell suspensions were subjected to a first centrifugation (360 g for 10 minutes at 20°C). When erythrocytes were not present, the supernatant was discarded and the pellet was resuspended. When present, erythrocytes were lysed by hypotonic shock by resuspending the cell pellet in *circa* 2.5 mL PBS and adding 7.5 mL of distilled water for 30 seconds. The cell suspension was then made up to 50 mL with further addition of PBS and a second centrifugation was performed to remove the cellular debris. The supernatant was discarded and the pellet resuspended in 5 mL PBS. in 5 mL PBS. Cell counts, viability assessment and cell identification were then performed.

Total cells retrieved in BALF were determined by counting the total number of cells in 1 mL BALF in a haemocytometer and multiplying the number of cells by the total volume of BALF recovered. Differential cell counts were performed from duplicate cytospin preparations of the original total cell suspensions following Wright-Giemsa staining. Approximately 400-600 cells were counted and the total number of cells of each type calculated. The results were presented as mean cell number ± S.E.M. (standard error of the mean).

Cell viability was assessed using the Trypan blue exclusion technique. Samples of the retrieved BALF (100 µL) were obtained, diluted with 20 µL Trypan blue and were counted in a haemocytometer. The total number of cells in the retrieved lavage fluid were extrapolated from the data obtained from the 100 µL samples.

5. HYPERREACTIVITY ASSAYS

Hyperreactivity assays were performed as follows: On each occasion, subsets of animals were sacrificed by exsanguination following pentobarbitone injection (40 mg • kg^{-1}). The trachea and lungs were quickly removed and placed in cold Krebs solution of the following composition (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.51, $MgSO_4 \cdot 7H_2O$ 1.18 and glucose 11.1. The right and left bronchi were gently dissected free of parenchyma and adhering connective tissue using a small metal rod (0.4-0.5 mm) as support. The lower bronchus was isolated and cut

into standardized spirals of approximately 1.5 mm width and 1.5 cm long. The diameter of the lower bronchi was 0.2-1.0 mm (Sirois *et al.*, 1987). The tissue preparation was installed in a cascade superfusion system and perfused at a rate of 5 $mL \cdot min^{-1}$ with oxygenated Krebs solution (95% O_2, 5% CO_2) maintained at 37°C. The preparations were left to equilibrate for 60 minutes under an initial loading tension of 1.5 g before bolus injections of histamine (9×10^{-10} to 4.5×10^{-6} mole) and acetylcholine (6.79×10^{-10} to 1.36×10^{-5} mole) (Sigma Chemical Co.,St-Louis, MO, U.S.A.) were initiated. Contractions were measured with Grass FT 03 C isometric force transducers and recorded on a Grass Polygraph (Model 7D).

6. STRUCTURAL EVALUATIONS

6.1 Histological Preparation

One, seven and 21 days following exposure completion, the guinea pigs allocated for histopathological evaluations were sacrificed by exsanguination following anaesthesia by i.p. injection of sodium pentobarbital. The lungs were dissected out of the thoracic cavity and were weighed in water; following taring of a beaker of water the lungs were inserted and the weight recorded. Assuming a tissue density of 1, the weight is equivalent to lung volume and allows the derivation of the volume of lung tissue accounted for by cells. The lungs were inflated using formalin vapors under pressure established from a 40 cm column of formalin. During preliminary experiments, column heights of 30 and 40 cm were examined and from histological sections prepared from animals processed following inflation under both sets of conditions, a height of the 40 cm was deemed most appropriate.

To allow thin sections to be prepared, lung samples were prepared in plastic (glycol methacrylate) rather than formalin using the JB-4 kit (PolySciences, Inc.). The lungs were retained in 10% neutral buffered formalin until they were trimmed. Trimming was conducted by cutting the lung into pieces small enough to fit into the embedding molds using a scalpel. Tissue samples were trimmed to be *circa* 1-2 mm thick and were placed into the molds such that they did not touch the sides of the mold. The sections were placed into 10% neutral buffered formalin until they were processed. Samples were subsequently dehydrated over a period of 4 hours by placing the samples sequentially into four baths of increasing denatured alcohol concentration at room temperature for

increasing periods of time as follows: 70% for 30 min, 95% for 30 min, 95% for 60 min, 100% for 60 min, 100% for 60 minutes. Following dehydration, lung samples were infiltrated by placing the sections into 100 mL of the JB-4 kit Catalyzed Solution A. This solution was prepared by measuring 100 mL of Solution A and adding 0.9 g of Catalyst C while mixing the solution on a magnetic stir plate. Sections were placed sequentially into two baths of Catalysed Solution A for 90 minutes each prior to placement into a third bath for approximately 16 hours (overnight).

Tissues were embedded into plastic glycol methacrylate in batches of less than 20 specimens at a time to ensure high quality samples. Glycol methacrylate was prepared by adding JB-4 Solution B to Catalyzed Solution A at a ratio of 1 part to 25 parts and mixing on a magnetic stir plate for 1 to 2 minutes. After the mixture had turned a straw color, the mixture was placed onto ice to slow the polymerization process. Following the addition of a small quantity of the embedding media to the mold, the lung samples were added to the embedding well. Each well cavity was subsequently filled with embedding media, the well was covered with an aluminum block holder in such a way as to minimize bubble formation and the block labelled. Additional embedding medium was pipetted through the hole in the top of the mold to completely fill the mold and the medium was allowed to polymerize. After polymerization was complete, the blocks were removed from the mold and were allowed to completely dry in a desicator.

Lung sections were prepared using a rotary microtome (AutoCut) fitted with glass knives. Following removal of sections of 10 µm in thickness to expose the tissue, the knife was changed and individual sections were cut at 1.0 µm thickness. To stretch the sections, individual sections were transferred onto a drop of water/alcohol solution (1 to 2 drops of alcohol to each 25 mL of water) and placed onto prelabelled, gelatin coated glass slides. These sections were dried at *circa* 50°C until the water had evaporated (*circa* 10 minutes).

Lung sections were stained with toluidine blue. A phosphate buffer was prepared by combination of 18 mL of Solution 1 (prepared by dissolving 27.6 g of $NaH_2PO_4 \cdot H_2O$ into 1 L of deionized water) with 81 mL of Solution 2 (prepared by dissolving 53.6 g of $NaH_2PO_4 \cdot 7H_2O$ into 1 L of deionized water). Following dissolution of 4 g of toluidine blue into 100 mL of the phosphate buffer, the final volume was brought to 200 mL with deionized water to provide 2% toluidine blue solution in 0.1 M phosphate buffer (pH 7.4). Sections were stained by applying the toluidine blue solution directly to the slides and rinsing the slides with distilled water 60 seconds later.

6.2 Histomorphometric Evaluation

In view of the extremely labor intensive nature of histomorphometric evaluation, an initial microscopic evaluation of all slides was performed. The purpose of this evaluation was to determine whether or not TDI exposure had resulted in the anticipated inflammatory changes and to serve as a basis of selection for airways in which histomorphometric evaluations would be made. During this evaluation, the diameter of the bronchioles in which infiltration was observed were determined for the purposes of selecting bronchioles of an appropriate diameter on which to perform quantitative measurements. Although the larger bronchioles tended to demonstrate more infiltration, the number of large bronchioles available was limited. In order to ensure an adequate number of bronchioles could be evaluated, the smallest bronchioles demonstrating infiltration were selected.

In order to perform a preliminary quantitative evaluation, representative slides were obtained from one representative control and TDI-exposed guinea pig sacrificed at each time point following exposure completion. Using a Leitz Orthoplan microscope equipped with a 10x eyepiece and 40x objective lenses, the bronchioles closest to 0.35 mm diameter (the length and width of the optical probe used) on each of the representative slides were selected. Because bronchioles are rarely observed to be round (ie. cut in exact cross section), the diameter was defined as the length of the smallest transect. In all cases, the bronchioles selected were between 0.25 and 0.42 mm (0.7 and 1.2 times the probe width). Furthermore, only those bronchioles in which the length was less than twice the diameter were used. The microscope was fitted with a Panasonic Digital 5000 camera which was connected to a MacIntosh Quadra A/V computer equipped with video image optimizing software (De-Babilizer). The video image was projected onto a monitor for measurements.

In each bronchiole, the probe was centered within the submucosa around the periphery of the bronchiole. Eight fields were selected from each bronchial beginning at "noon" and proceeding clockwise around the bronchiole at 45° intervals as indicated in Figure 6.

Correcting for magnification, each field was 0.097 mm x 0.097 mm. Even in the smallest diameter bronchioles (0.25 mm), the eight fields did not overlap. Submucosal measurements were made using commercial histomorphometry software (Morphometrix, Inc., Stereology Toolbox™).

The ratio of infiltrating cell volume to submucosal surface area was assessed using the "point counting" method. These measurements were made by overlaying a grid onto the projected image

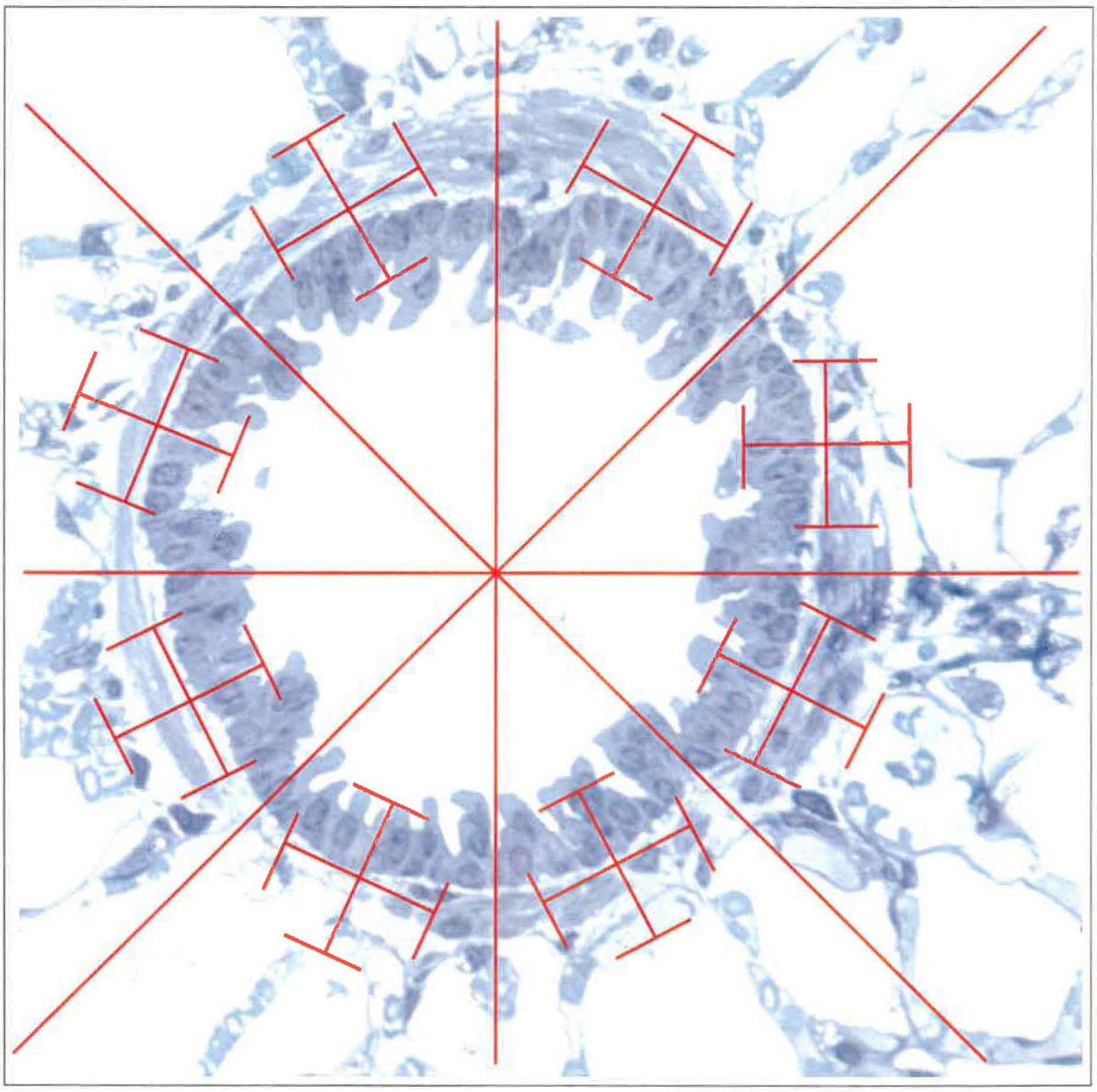


FIGURE 6 - Schematic Representation of Fields Used for Histomorphometric Analysis.

and counting the number of grid points which fell over infiltrating cells and those which fell over submucosal tissue. The results were expressed as total cell volume per unit surface area. Although the cells counted comprised macrophages, monocytes, eosinophils and neutrophils and could have been separated by type, the most reliable data used to support inflammation was considered to be the use if the total cell numbers. The subdivision of cell types was considered inappropriate as a result of decreased reliability with lower numbers of cells.

7. STATISTICAL ANALYSIS

The experiments reported herein were originally intended to be followed by further investigations in which guinea pigs were treated with pharmacological agents to attempt to block or inhibit effects of TDI exposure. Due to the volume of work required to assess the inflammatory changes, bronchial hyperreactivity, and histopathological and morphometric findings observed in these experiments, these additional investigations were not performed. Statistical analysis of the data obtained was desirable, but in view of the limited number of control animals (N=2 per experiment), the reliable application of statistical procedures required careful selection of tests. Due to the design of the experiment, the approach selected was analysis by 2 way ANOVA. The 2 way ANOVA is ideal for the comparisons required in studies of this design as it creates and uses a common group variance which markedly increases the reliability of the results of the analysis despite the low number of control animals. When compared with a one way ANOVA, the increased reliability (measured in degrees of freedom) of the 2 way ANOVA is evident: using a one way ANOVA, 4 to 5 degrees of freedom are available; with the 2 way ANOVA, 17 degrees of freedom are available.

As a prerequisite to the reliable use of the 2 way ANOVA, the variance within the control and TDI-exposed animal data collected 1, 7 and 21 days post-exposure must be homogeneous. The homogeneity of variance was established using Bartlett's test. Statistical significance was declared at $P \leq 0.05$ for the 2 way ANOVA and at $P < 0.001$ for Bartlett's test.

7.1 Bronchoalveolar Lavage Data

Statistical analysis was performed to assess the effect of TDI-exposure on the total number of cells retrieved in BALF. In view of the wide variability in the numbers of cells retrieved, statistical comparisons of the numbers of macrophages, eosinophils and neutrophils retrieved in BALF were performed on values which were normalized as a percentage of the total cells retrieved. The significance of TDI-exposure on the total number of cells recruited into the airways as well as the proportion of cells represented by macrophages, eosinophils and neutrophils cells was determined using the following comparisons:

1. Effect of TDI-Exposure Within Each Day: The effect of TDI exposure on the number (percentage) of cells recovered within each day was determined by comparing the data collected from control and TDI-exposed animals sacrificed on the same day, ie. cell counts obtained from control and TDI-exposed animals 1 day after exposure completion were compared; similar comparisons were performed on data collected 7 and 21 days after exposure completion. A significant Exposure effect indicates that TDI-exposure resulted in a significant change in the number (percentage) of cells retrieved in the BALF.

2. Effect of Time on TDI-Exposure: The effect of TDI exposure on the number (percentage) of cells recovered within each group but between days was assessed by comparing the number (percentage) of cells recovered from TDI-exposed animals sacrificed 1, 7 and 21 days post-exposure, ie. the data collected from TDI-exposed animals on Day 1 was compared to the data collected from TDI-exposed animals on Day 7 as well as the data collected on Day 21; in addition, the data collected from TDI-exposed animals on Day 7 was compared to that collected on Day 21. Similar comparisons were made within the control data. A significant Time effect indicates that the duration of the recovery period has an important effect on the number (percentage) of cells retrieved in the BALF.

Because the variance was homogeneously distributed, untransformed total cell counts were analyzed. The variance within the macrophage and eosinophil data was not homogeneously

distributed and therefore the data was transformed to obtain homogeneity within the variance to permit analysis using the 2 way ANOVA. Homogeneity of variance was established using ARCSIN transformed percentage macrophage and eosinophil data. Similarly, the variance within the untransformed neutrophil counts was not homogeneous. Homogeneity of variance was established following logarithmic transformation and subsequent analysis was conducted as detailed above.

In all cases (untransformed total cell counts, ARCSIN transformed macrophage and eosinophil percentages and logarithmically transformed total and percentage neutrophil counts), the homogeneity of group variance was established using Bartlett's test (not significant, $P<0.001$).

7.2 Hyperreactivity Data

In order to assess the significance of TDI-exposure on bronchial reactivity to agonist challenge, statistical analyses were performed to compare the areas under the dose-response curves (AUC) constructed from TDI-exposed guinea pigs to those obtained from air controls. AUCs were calculated for each animal individually over the range of histamine and acetylcholine concentrations by applying the trapezoidal rule to individual (contractile response, agonist concentration) data pairs. Although individual experiments were performed separately on the left and right bronchi, because both bronchi were obtained from the same animal, it was considered more appropriate to regard the left and right data together rather than separately. As such, a third variable, termed TOTAL RESPONSE (TOTAL) was constructed by summing the contractile force exerted by the left and right bronchi at each agonist concentration. The TOTAL AUC was determined for each animal individually by applying the trapezoidal rule to the summed right and left bronchi contractile response at each agonist concentration.

Statistical evaluation of the effect of TDI-exposure on bronchial contraction was performed by applying the 2 way ANOVA to the TOTAL AUC dose-response curves constructed following histamine and acetylcholine challenge. In this test, the effect of two variables (Exposure effect and Time effect) on the contractile response were separately considered:

1. Effect of TDI-Exposure Within Each Day: The effect of TDI exposure on the contractile response within each day was assessed by comparing the TOTAL AUC calculated from control

and TDI-exposed animals sacrificed on the same day (ie. TOTAL AUC calculated from control and TDI-exposed animals 1 day after exposure completion were compared; similar comparisons were performed on data collected 7 and 21 days after exposure completion). A significant Exposure effect indicates that TDI-exposure resulted in a significant change in the magnitude of bronchial hyperreactivity.

2. Effect of Time on TDI-Exposure: The effect of TDI exposure on the contractile response within each group but between days was assessed by comparing the TOTAL AUC calculated from TDI-exposed animals sacrificed 1 day after exposure completion with the corresponding data collected 7 and 21 days after exposure completion. In addition, the data collected from TDI-exposed guinea pigs 7 days after exposure completion is compared with the corresponding data collected 21 days after exposure completion (similar comparisons are made within the control data). A significant Time effect indicates that the duration of the recovery period is important in the magnitude of the bronchial hyperreactivity.

In all cases (untransformed left bronchi AUC, right bronchi AUC and TOTAL AUC), the homogeneity of group variance was established using Bartlett's test (not significant, $P<0.001$). The data obtained from the untransformed TOTAL AUC data were used to assess statistical significance.

RESULTS

CHAPTER 5

RESULTS

1. INHALATION EXPOSURE

The mean chamber concentrations (with associated standard errors of the mean, S.E.M.) measured over the 5 days of the study during guinea pig exposure exposures were the following:

TABLE NO. 3: TDI Inhalation Chamber Vapor Concentrations (ppm)

Exposure Day	Exposure Hour				Mean	S.E.M.
	1	2	3	4		
1	1.1	2.7	2.6	3.2	2.4	0.5
2	2.0	3.1	4.4	3.2	3.2	0.5
3	2.0	3.0	3.0	3.9	3.0	0.4
4	9.5	2.3	1.5	2.4	3.9	1.9
5	0.8	1.5	2.3	3.6	2.0	0.6
		Overall Mean ± S.E.M.			2.9	0.4

Due to variation in the concentration of TDI vapor output from the vapor generator, initial TDI vapor concentrations were more variable than expected from preliminary investigations. However, over the 5 days of exposure, the mean TDI concentrations to which the guinea pigs were exposed were appropriate to elicit the desired response. The variability in chamber concentrations were therefore considered not to have any impact on the interpretation of the data obtained from this study.

Analysis of the sample collected from the air control chamber during the fourth day of exposure indicated the presence of TDI at a concentration of 0.02 ppm. When considered in conjunction with the absence of similar results during prestudy atmosphere characterization work and the remaining 4 days of exposure, the isolated observation of trace level contamination of the air control atmosphere was attributed to contamination of one of the reactants with TDI and was considered not to be representative of the atmosphere to which the air control animals were exposed on Day 4.

2. BRONCHOALVEOLAR LAVAGE

2.1 Total Number of Cells Recovered in BALF

Bronchoalveolar lavage (BAL) provided evidence of a marked infiltration of leukocytes into the airways following 5 days of exposure to TDI (Figure 7). When compared with concurrent controls, a greater than four fold increase in total number of cells recovered in bronchoalveolar lavage fluid (BALF) was observed one day after the completion of TDI exposure. Seven days post-exposure the total number of cells recruited into BALF had decreased to approximately twice the concurrent control values while 21 days after exposure completion approximately equivalent numbers of cells were retrieved in BALF.

Statistical comparisons were performed on untransformed total cell counts. As expected, statistically significant ($P \leq 0.05$) interaction between Time and Exposure was demonstrated (ie. the relationship between the number of cells recruited into control and TDI-exposed animals was dependant upon the day the animals were sacrificed). As such, data collected 1, 7 and 21 days post-exposure could not be combined and each comparison was treated separately to determine the effect of Time on total number of cells recovered within air control and TDI-exposed populations (ie. determination of statistical differences between cells recovered from TDI-exposed animals: 1 day *vs.* 7 days, 1 day *vs.* 21 days and 7 days *vs.* 21 days and similar comparisons within air control animals) and the effect of TDI-exposure on cell counts (ie. comparison of air control and TDI data obtained on each day).

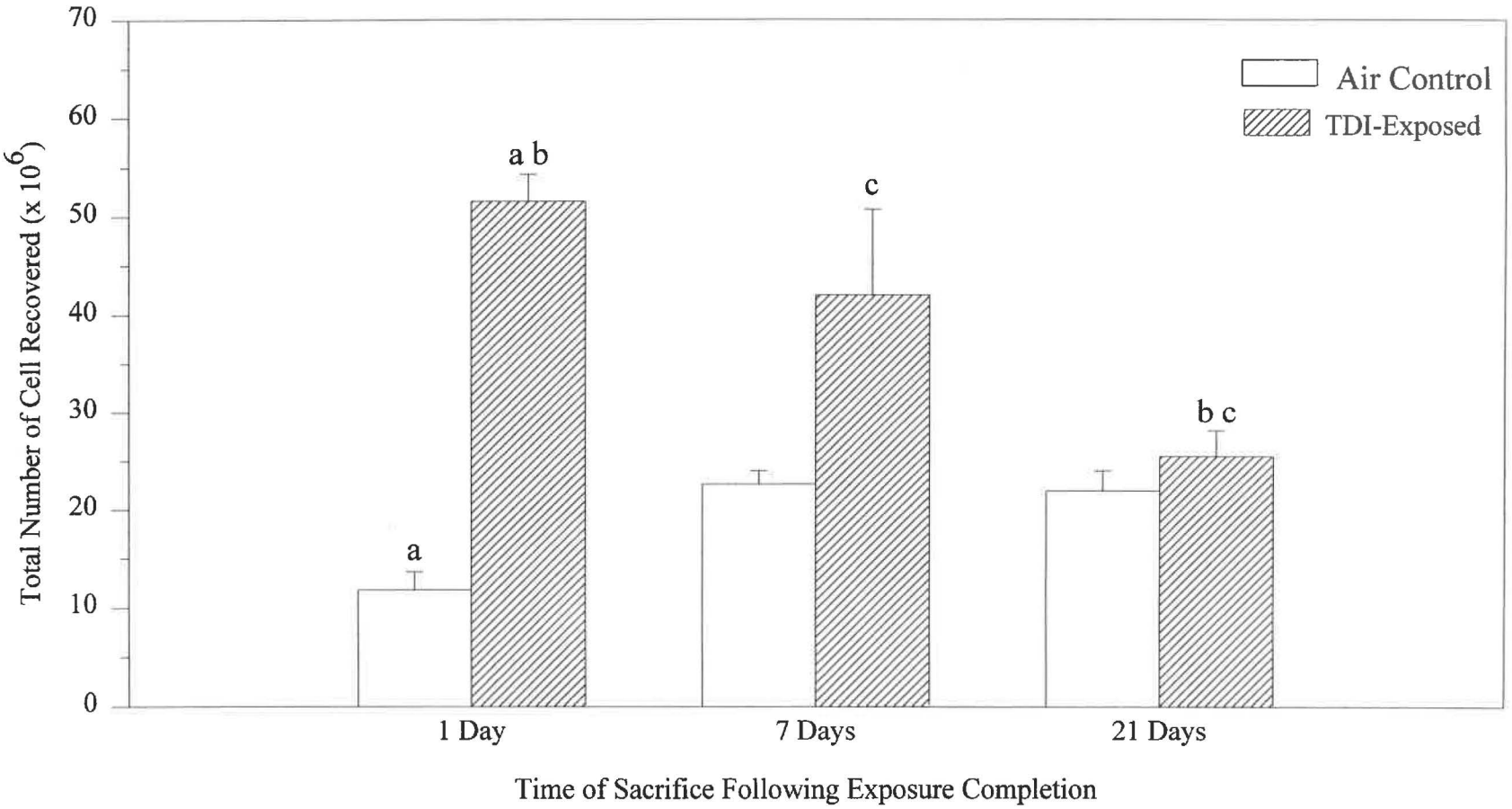
There were no statistically significant differences between control data collected 1, 7 and 21 days post-exposure. When compared to controls sacrificed on the same days, the total number of cells recovered from TDI-exposed guinea pigs were significantly higher 1 day after exposure ($P=0.0006$), but not 21 days after exposure ($P=0.80$). Although the cell counts obtained 7 days after exposure from TDI-exposed guinea pigs were higher than controls on the same day, the results were not significantly different from those obtained 1 day after exposure ($P=0.06$).

Comparison of total number of cell recovered in BALF of TDI-exposed guinea pigs 1, 7 and 21 days post-exposure yielded the following results: the total number of cells recovered 1 and 7 days after exposure were not statistically different ($P=0.19$); significantly lower numbers of cells were

Figure No. 7

Total Numbers of Leukocytes Recovered in Bronchoalveolar Lavage Fluid

(Mean ± S.E.M.)



Data indicated by the same letter are significantly different ($P<0.05$).

recovered from TDI-exposed guinea pigs 21 days after exposure than were recovered 1 day post-exposure (P=0.0008); significantly lower numbers of cells were recovered from TDI-exposed guinea pigs 21 days after exposure than were recovered 7 days after exposure (P=0.02).

2.2 Changes in Cell Populations Recovered in BALF

In addition to marked infiltration of total numbers of leukocytes, BALF results provided evidence of changes in the proportional representation of cell populations recovered from the airways of air and TDI-exposed guinea pigs. Following cytospin preparation and subsequent Wright-Giemsa staining, differential cell counts were performed to determine the relative numbers of macrophages, eosinophils and neutrophils. When compared with corresponding controls, eosinophils and neutrophils increased 20 and 50 fold while macrophage counts decreased two fold one day after exposure completion (Table 4).

Macrophage counts include monocytes (identified by the lack of cytoplasmic vacuoli), fully vacuolized macrophages and alveolar macrophages due to the difficulty in segregating populations of these cell types with these techniques. Similarly, as it was difficult to identify lymphocytes under these conditions, lymphocyte populations were not counted.

TABLE NO: 4 Group Mean (S.E.M.) Absolute Leukocyte Counts Recovered From Bronchoalveolar Fluid

Day of Sacrifice	Treatment	Total Number of Cells Recovered ($x10^6$)	Mean (±S.E.M.) Absolute Leukocyte Counts ($x10^6$)		
			Macrophages	Eosinophils	Neutrophils
1	Air Control	11.82 ± 1.86	10.90 ± 1.97	0.73 ± 0.04	0.19 ± 0.07
	TDI-Exposed	51.56 ± 2.77	25.45 ± 2.23	15.97 ± 2.22	10.14 ± 4.49
7	Air Control	22.62 ± 1.38	20.54 ± 1.68	1.96 ± 0.27	0.11 ± 0.04
	TDI-Exposed	42.02 ± 9.53	24.35 ± 5.54	15.66 ± 3.28	2.03 ± 1.71
21	Air Control	21.96 ± 2.04	19.30 ± 3.02	2.58 ± 0.90	0.09 ± 0.09
	TDI-Exposed	24.36 ± 2.64	18.56 ± 1.79	5.31 ± 1.19	0.49 ± 0.19

2.2.1 Macrophages

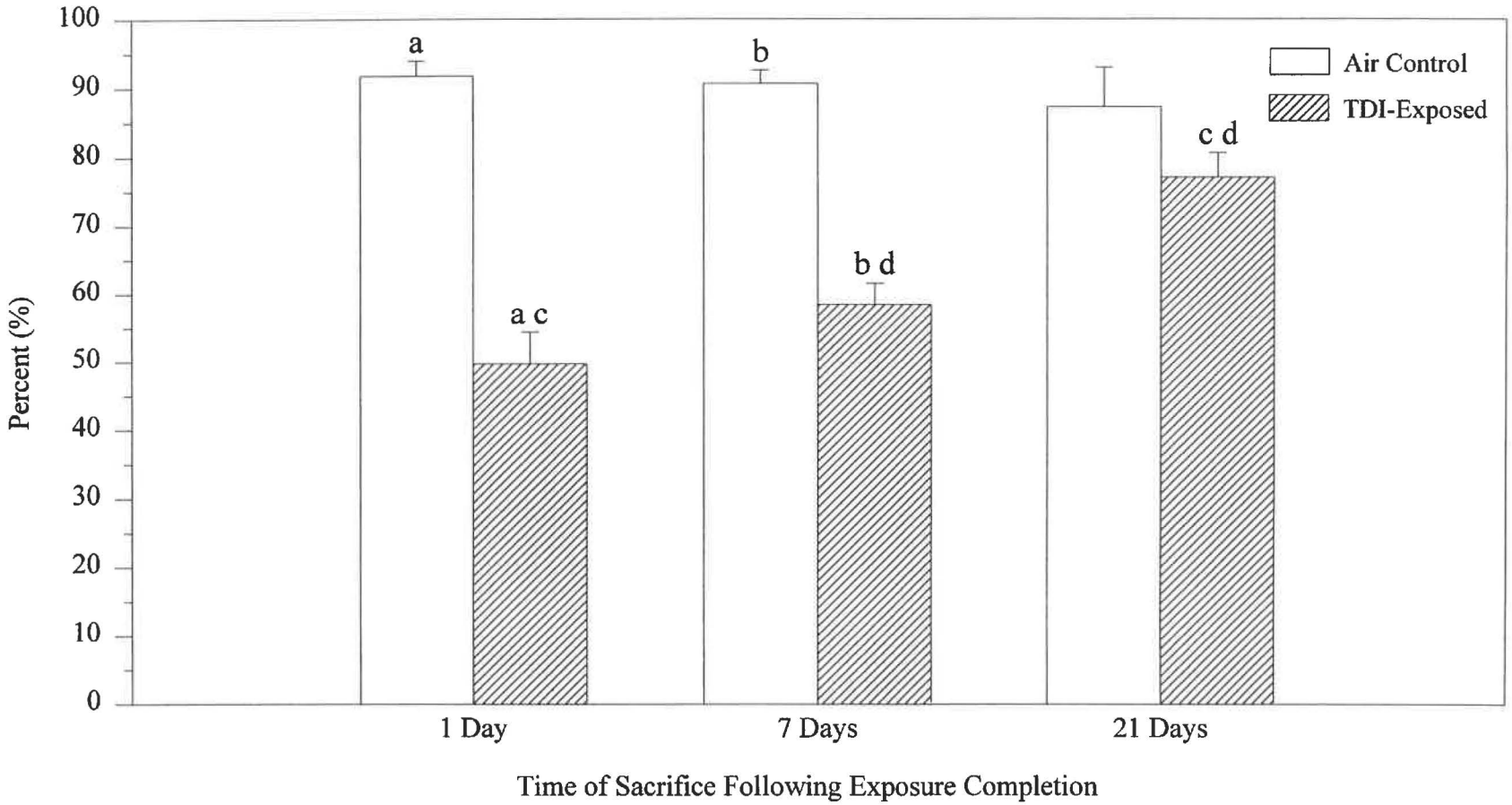
Macrophage counts obtained 1, 7 and 21 days after completion of TDI-exposure are presented as a percentage of the total cells recovered in BALF in Figure 8. Significant interaction (P=0.02) between Time and Exposure was demonstrated and therefore comparisons of data collected 1, 7 and 21 days post-exposure were performed separately. Macrophage data collected from air controls sacrificed 1, 7 and 21 days post-exposure were not significantly different. When compared to controls sacrificed on the same days, TDI-exposure resulted in a statistically significant decrease in macrophage numbers 1 day and 7 days post-exposure (P=0.0001 and P=0.0002, respectively). Twenty-one days post-exposure BALF recovered macrophage numbers obtained from air control and TDI-exposed animals were statistically similar (P=0.12).

Comparison of macrophage data obtained from TDI-exposed animals over time indicated that although the difference between macrophage numbers recovered 1 and 7 days post-exposure were not significant (P=0.17), significantly higher numbers of macrophages were recovered 21 days post-exposure than were recovered either 1 or 7 days post-exposure (P=0.0001 and P=0.003, respectively).

Figure No. 8

Macrophages Recovered in Bronchoalveolar Lavage (% Total Cells)

(Mean ± S.E.M.)



Data indicated by the same letter are significantly different ($P<0.05$).

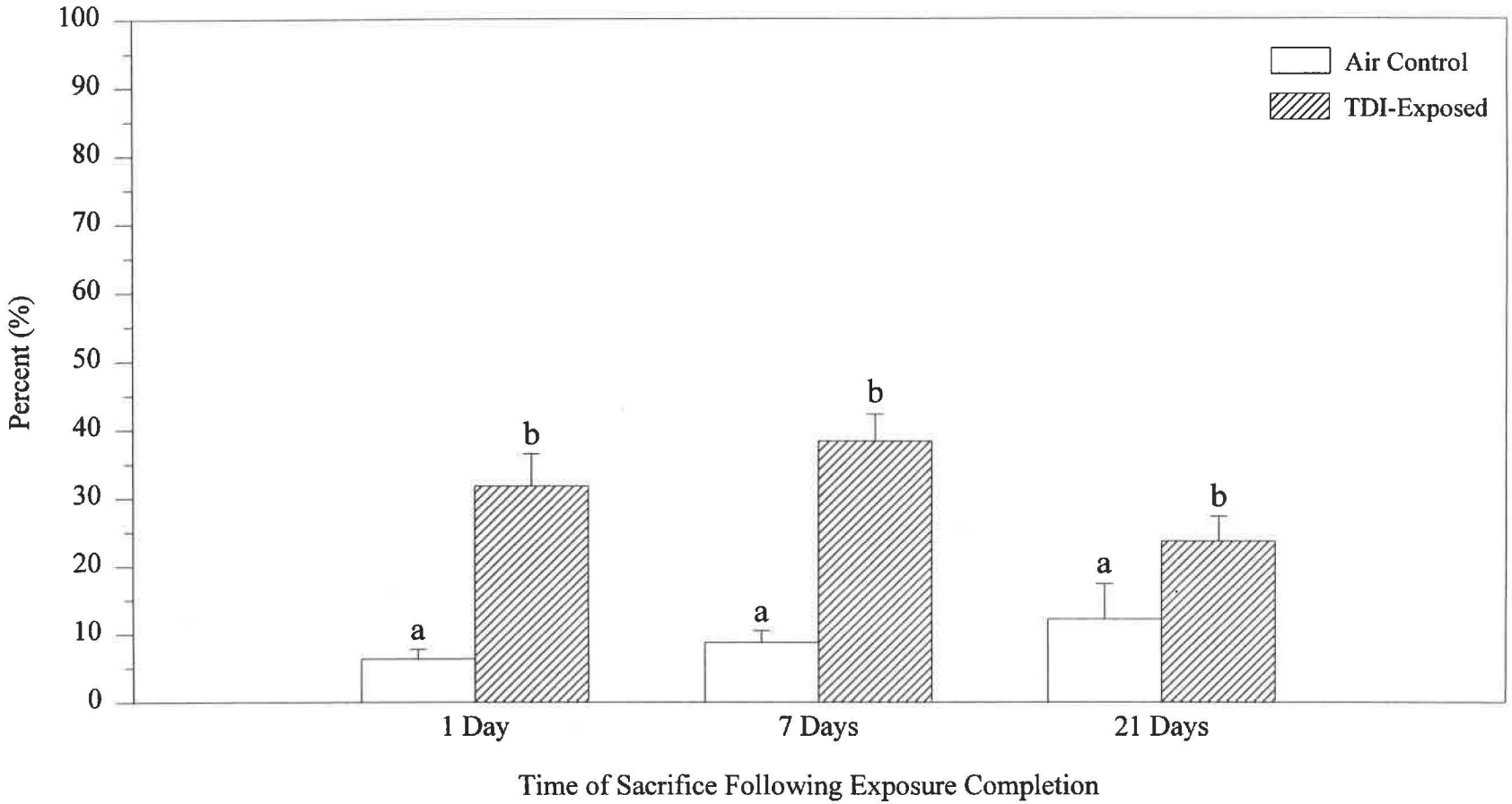
2.2.2 Eosinophils

Eosinophil counts obtained 1, 7 and 21 days after completion of TDI-exposure are presented as a percentage of the total cells recovered in BALF in Figure 9. Statistical comparisons of the numbers of eosinophils recovered in BALF were performed on ARCSIN transformed percentages of total cells represented by eosinophils. In the absence of significant interaction between Time and Exposure effects (P=0.08), comparison of the combined data collected from control and TDI-exposed animals was performed. These analyses indicated that numbers of eosinophils recovered from TDI-exposed animals was significantly higher than from the controls (P=0.0003).

Figure No. 9

Eosinophils Recovered in Bronchoalveolar Lavage (% Total Cells)

(Mean ± S.E.M.)



a, b: Combined Air Control data is significantly different from the combined TDI-Exposed data ($P<0.05$)

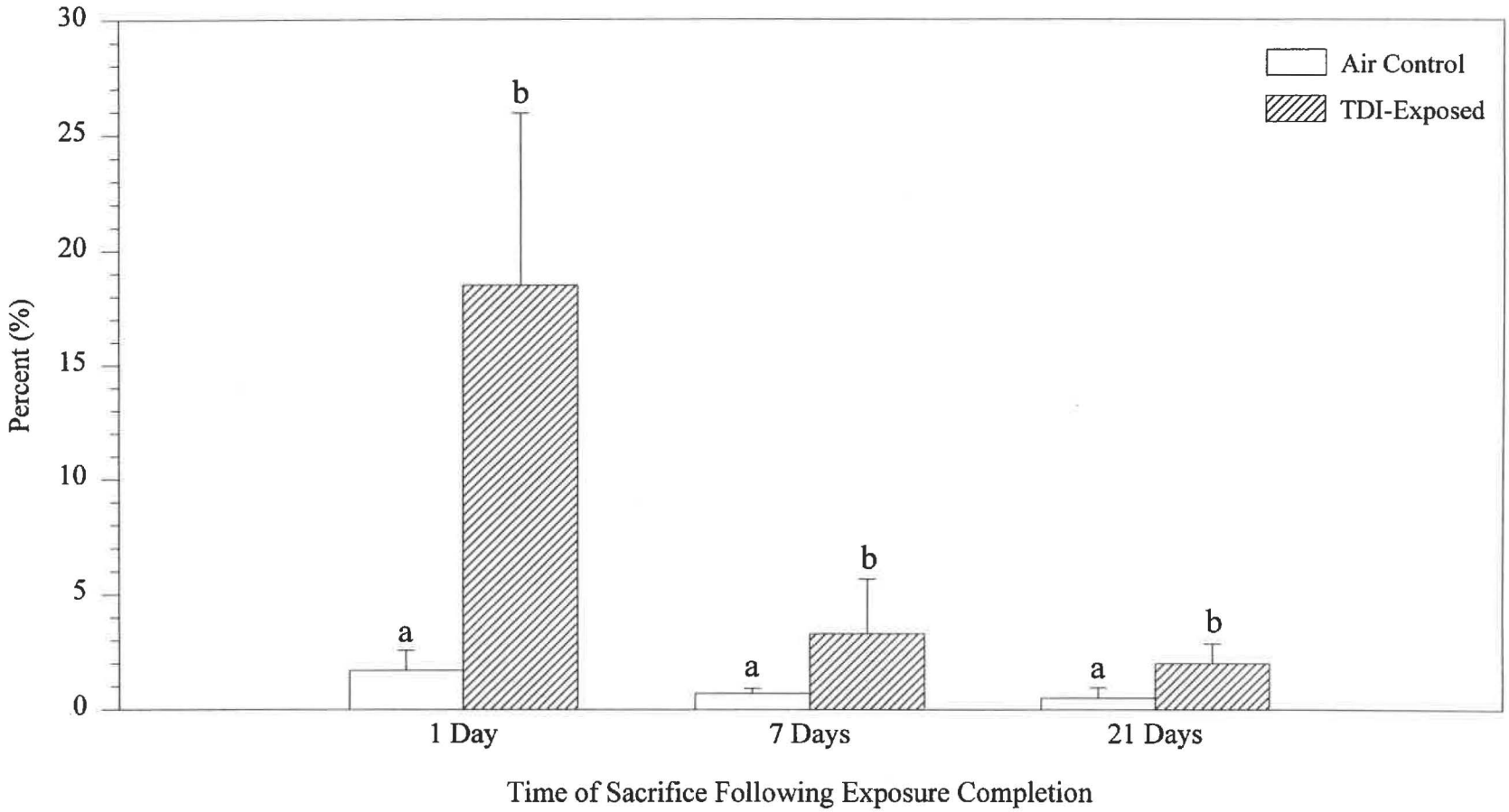
2.2.3 Neutrophils

Neutrophil counts obtained 1, 7 and 21 days after completion of TDI-exposure are presented as a percentage of the total cells recovered in BALF in Figure 10. Statistical comparisons of the numbers of neutrophils recovered in BALF were performed on logarithmically-transformed total neutrophil counts. In the absence of significant interaction between time and exposure effects (P=0.16), comparison of the combined data collected from control and TDI-exposed animals was performed. These analyses indicated that numbers of neutrophils recovered from TDI-exposed animals was significantly higher than from the controls (P=0.02).

Figure No. 10

Neutrophils Recovered in Bronchoalveolar Lavage (% Total Cells)

(Mean ± S.E.M.)



a, b: Combined Air Control data is significantly different from combined TDI-exposed data ($P<0.05$).

3. HYPERREACTIVITY ASSAYS

3.1 Bronchial Hyperreactivity Data

Agonist challenge of isolated bronchi provided marked evidence of bronchial hyperreactivity following 5 days of exposure to TDI. Both control and TDI-exposed bronchi responded to agonist challenge over the full range of concentrations tested (0.1 to 1500 µg acetylcholine and 0.1 to 200 µg histamine). Maximum responses to histamine and acetylcholine in TDI-exposed bronchial strips were obtained at doses of 50 or 100 µg histamine and 1000 µg acetylcholine. Responses were most pronounced the day after exposure completion. When expressed in terms of the TOTAL AUC constructed from the summed right and left bronchi responses, the responses 1 day after exposure were approximately 3 to 4 times that observed 7 and 21 days post-exposure. Responses observed 7 and 21 days post-exposure were similar and were approximately two to three times the concurrent control values.

Statistical comparisons were performed on the basis of TOTAL AUC values. Significant Time*Exposure interaction was observed following histamine challenge (P=0.04). Although interaction between Time and Exposure was not statistically significant at the P=0.05 level following acetylcholine challenge (P=0.07), the difference between the probability that this difference arose by chance (ie. P=0.07) and the arbitrary selection of P=0.05 as the level for declaring statistical significance was considered marginal. Because the lack of significant Time*Exposure interaction implied the magnitude of the difference in contractile force following acetylcholine challenge was related to either TDI exposure or the duration of the recovery period following exposure completion rather than both parameters, and when considered along with the significant difference in TOTAL AUC following histamine challenge, the acetylcholine TOTAL AUC Time*Exposure interaction was considered significant for the purposes of subsequent statistical evaluations.

The effect of Treatment (TDI-exposure) on bronchial reactivity to agonist challenge was evaluated within each day (ie. comparison of air control and TDI data obtained on each day) and the effect of Time was evaluated by comparing the responses of TDI-exposed animals between each day (ie. determination of statistical differences in bronchial response to agonist challenge of TDI-exposed animals: 1 day *vs.* 7 days, 1 day *vs.* 21 days and 7 days *vs.* 21 days and similar comparisons within air control animals). All comparisons were made on the basis of TOTAL AUC.

Results of acetylcholine challenge (ACh) were identical to histamine (His) challenge. Comparison of TOTAL AUC from air control animals sacrificed 1, 7 and 21 days post-exposure yielded no statistically significant differences. When compared to controls sacrificed on the same day, the TOTAL AUCs obtained from TDI-exposed guinea pigs were strongly significantly greater 1 day after exposure (ACh: P=0.0002; His P=0.0005) and were statistically similar 7 and 21 days after exposure (7 days: ACh: P=0.13; His P=0.10; 21 days: ACh: P=0.16; His P=0.18).

Comparison of TOTAL AUCs obtained from TDI-exposed guinea pigs 1, 7 and 21 days post-exposure indicated that responses obtained 1 day after exposure were significantly greater than either 7 or 21 days post-exposure (ACh: P=0.0002 and 0.0004, His: P=0.0001 and P=0.0001, respectively). Responses obtained 21 days after exposure completion were not statistically different from those obtained 7 days post-exposure (ACh: P=0.71; His: P=0.90).

The increased force of contraction was reflected in the qualitative observation of an increase in the slope of the log dose-response curves of TDI-exposed bronchi to histamine and acetylcholine challenge.

Figure No. 11

Effect of TDI on Histamine-Induced Contraction of Bronchial Strips

(Mean ± S.E.M.)

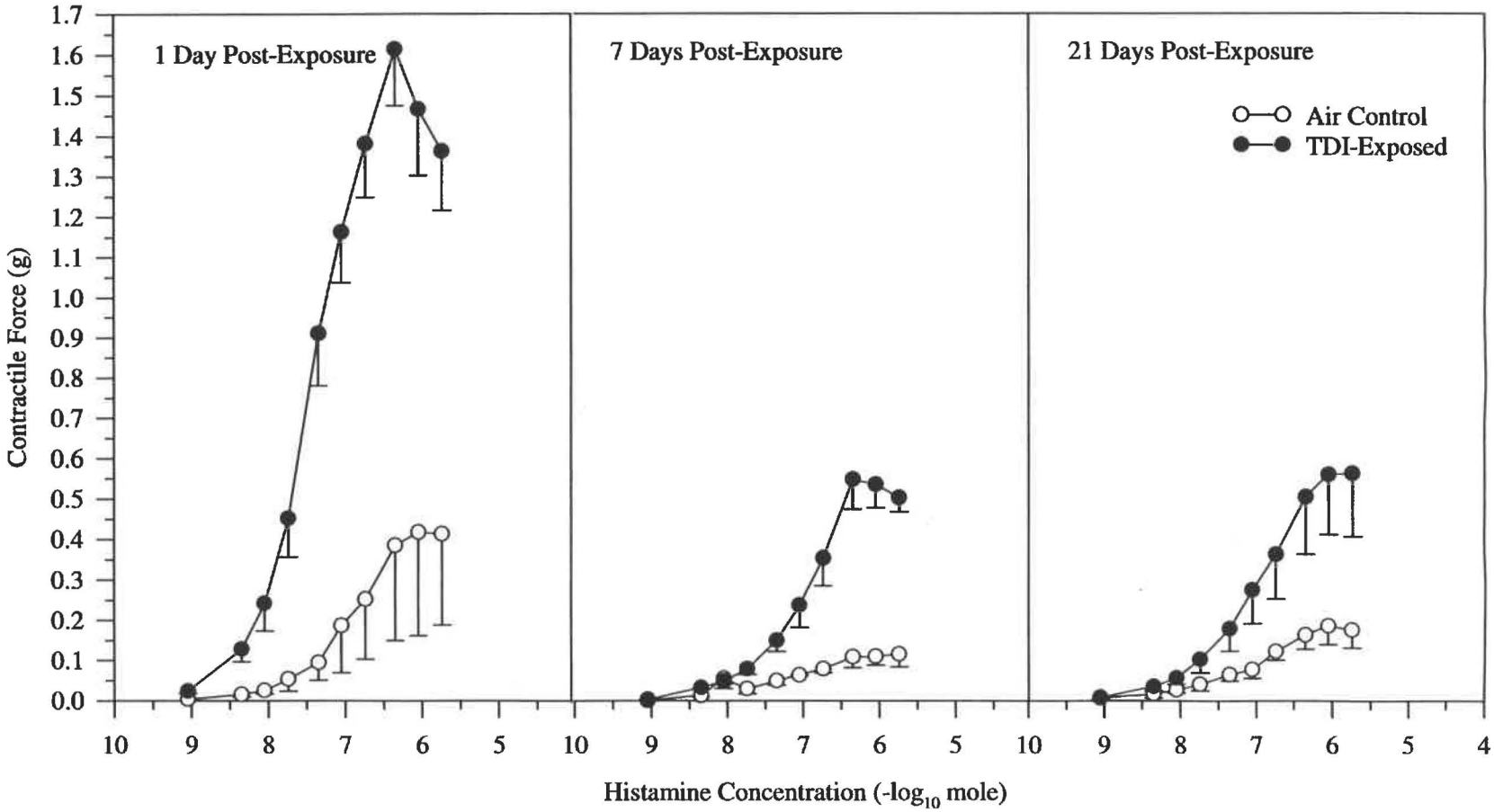
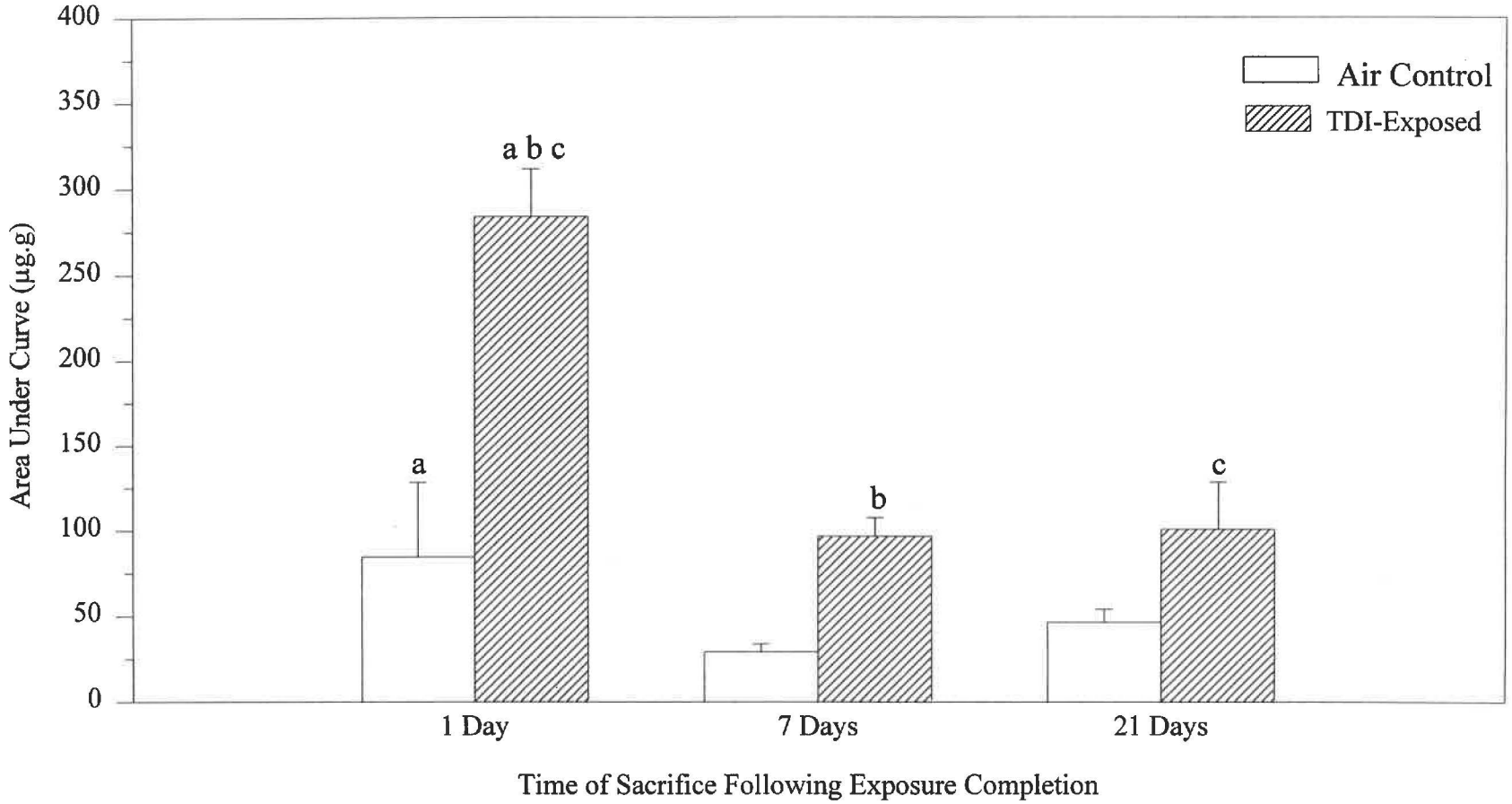


Figure No. 12

Effect of TDI on Histamine-Induced Contraction of Bronchial Strips

(Mean ± S.E.M.)



Data indicated by the same letter are significantly different ($P<0.05$).

Figure No. 13

Effect of TDI on Acetylcholine-Induced Contraction of Bronchial Strips

(Mean ± S.E.M.)

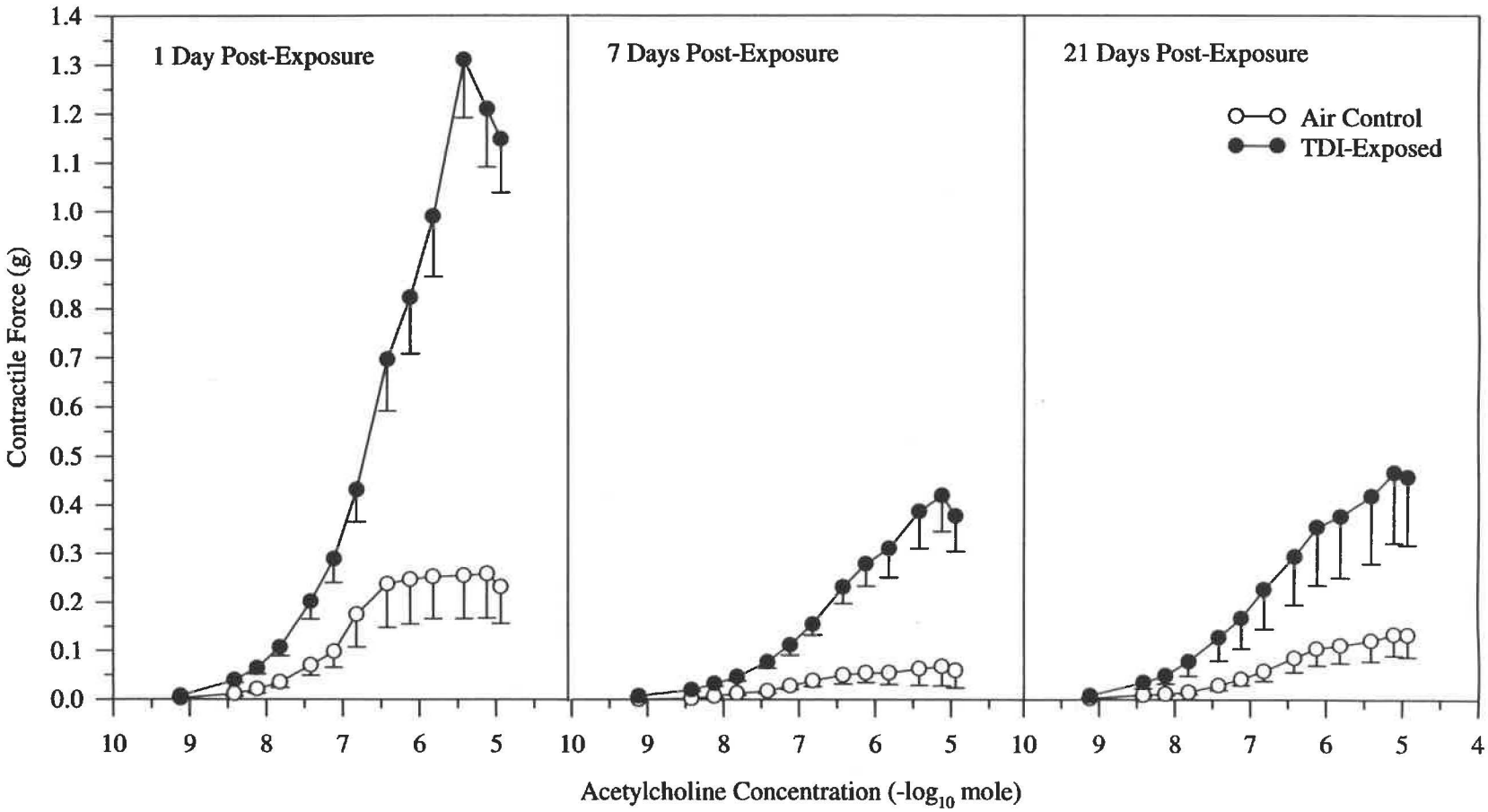
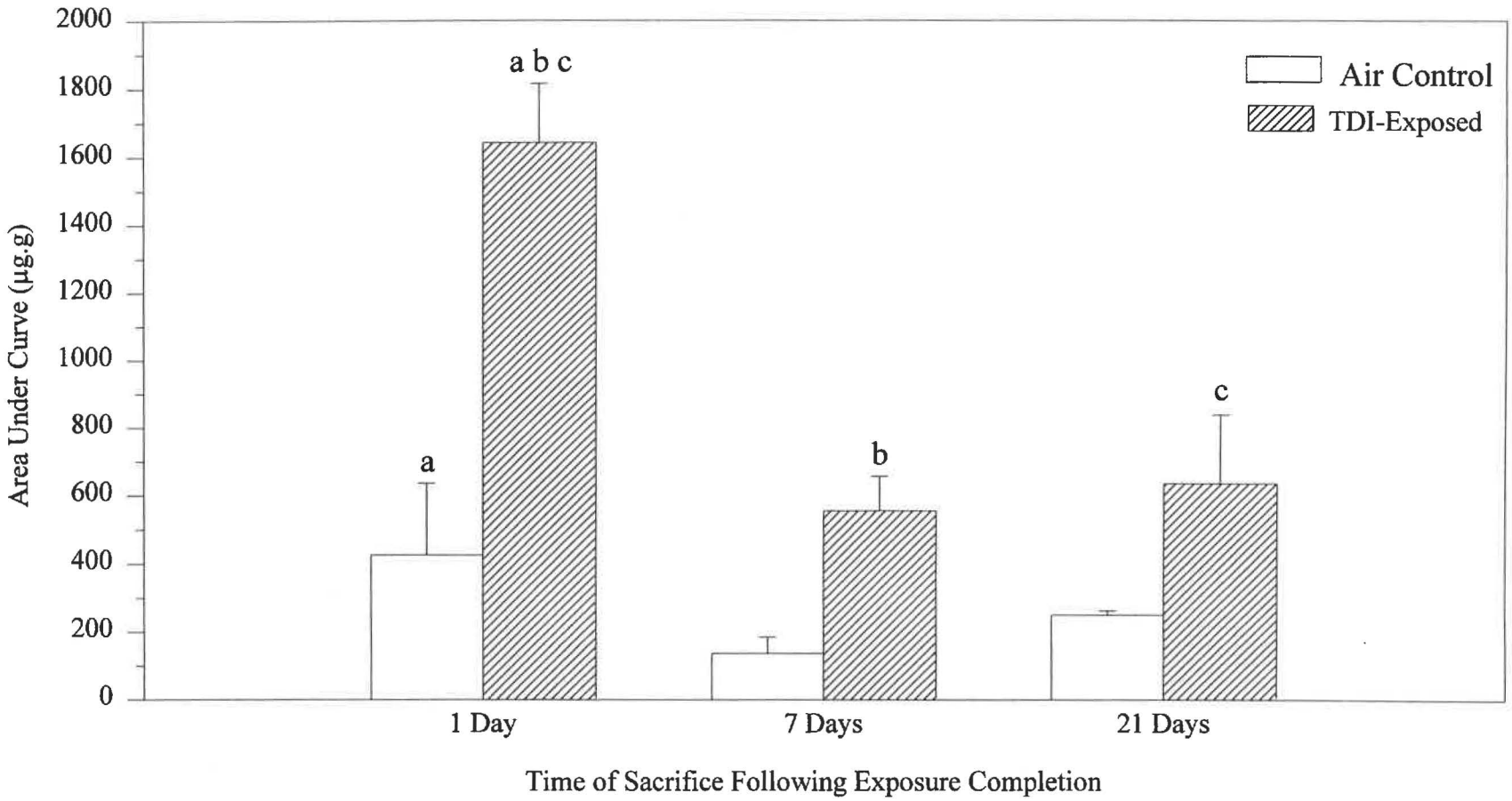


Figure No. 14

Effect of TDI on Acetylcholine-Induced Contraction of Bronchial Strips

(Mean ± S.E.M.)



Data indicated by the same letter are significantly different ($P<0.05$).

4. STRUCTURAL EVALUATIONS

4.1 Histopathological Evaluation

Qualitative evaluation of all sections obtained from guinea pigs sacrificed 1, 7 and 21 days after exposure completion were performed. Representative sections are presented in the composite plate illustrated in Figure 15. The four photomicrographs presented in this composite plate were obtained at magnifications of 250x. These photomicrographs were representative of the histopathological appearance of the bronchial sections obtained from air control animals (1), and animals sacrificed 1 day (2), 7 days (3) and 21 days (4) following TDI-exposure completion.

When compared with air controls, histopathological changes observed 1 day following exposure completion clearly indicated epithelial damage characterized by epithelial sloughing (2Ⓐ→), replacement of the normal pseudostratified, ciliated columnar epithelium by stratified cuboidal epithelioid cells (2Ⓑ→), clear submucosal inflammation (2Ⓒ→), and edema (Figure 15-1 *vs.* 15-2). Seven days after exposure completion, the beginnings of repair were evident and were characterized by lack of observation of epithelial sloughing (Figure 15-3). While submucosal inflammation was still apparent (3Ⓐ→), it was present to a lesser extent than was observed the day following exposure completion. Similarly, the epithelium had not yet returned to the normal pseudostratified, ciliated columnar appearance (3Ⓑ→). Slides prepared from animals sacrificed 21 days after exposure completion indicated obvious epithelial regeneration and a relatively normal histological appearance (Figure 15-4).

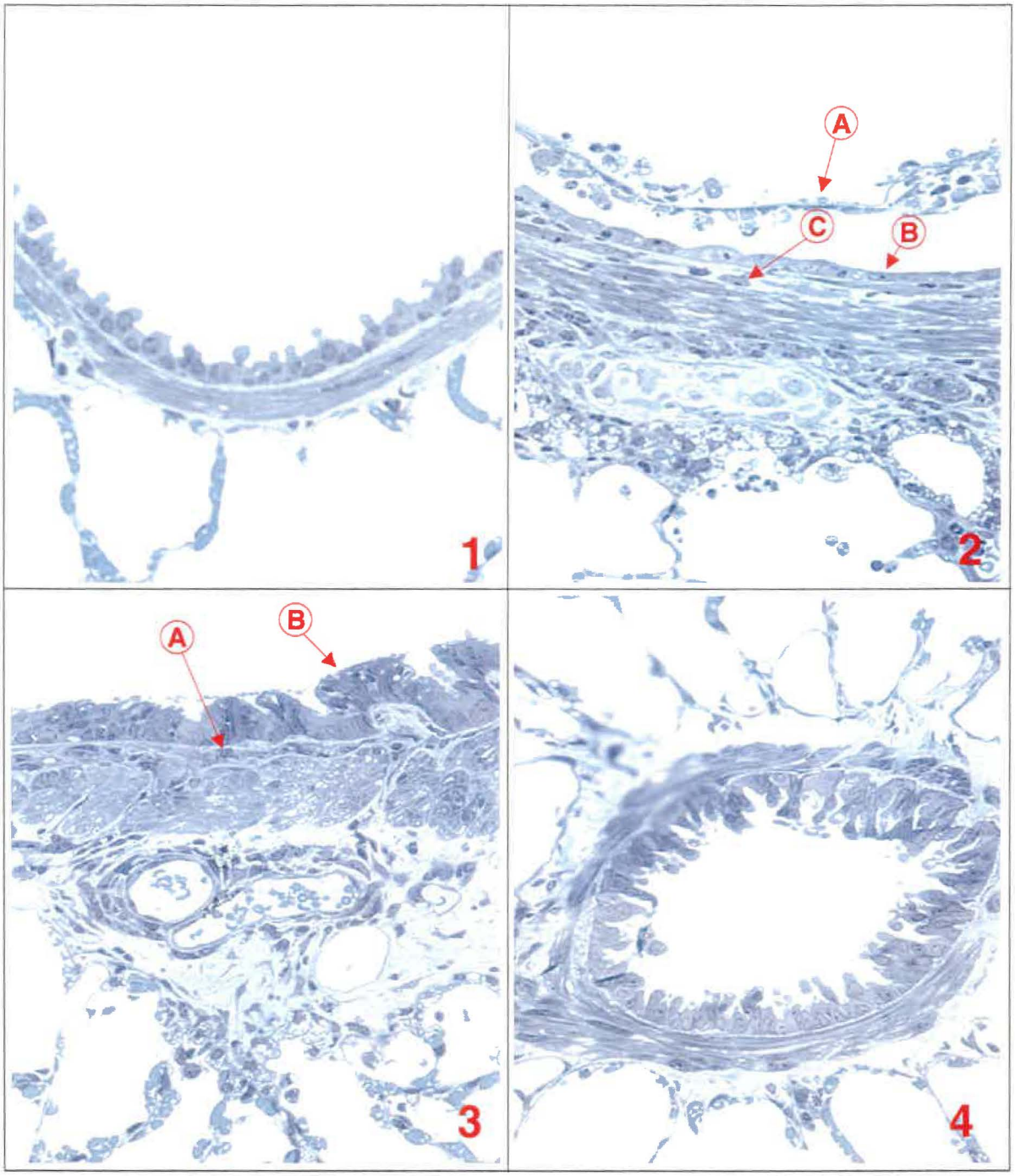


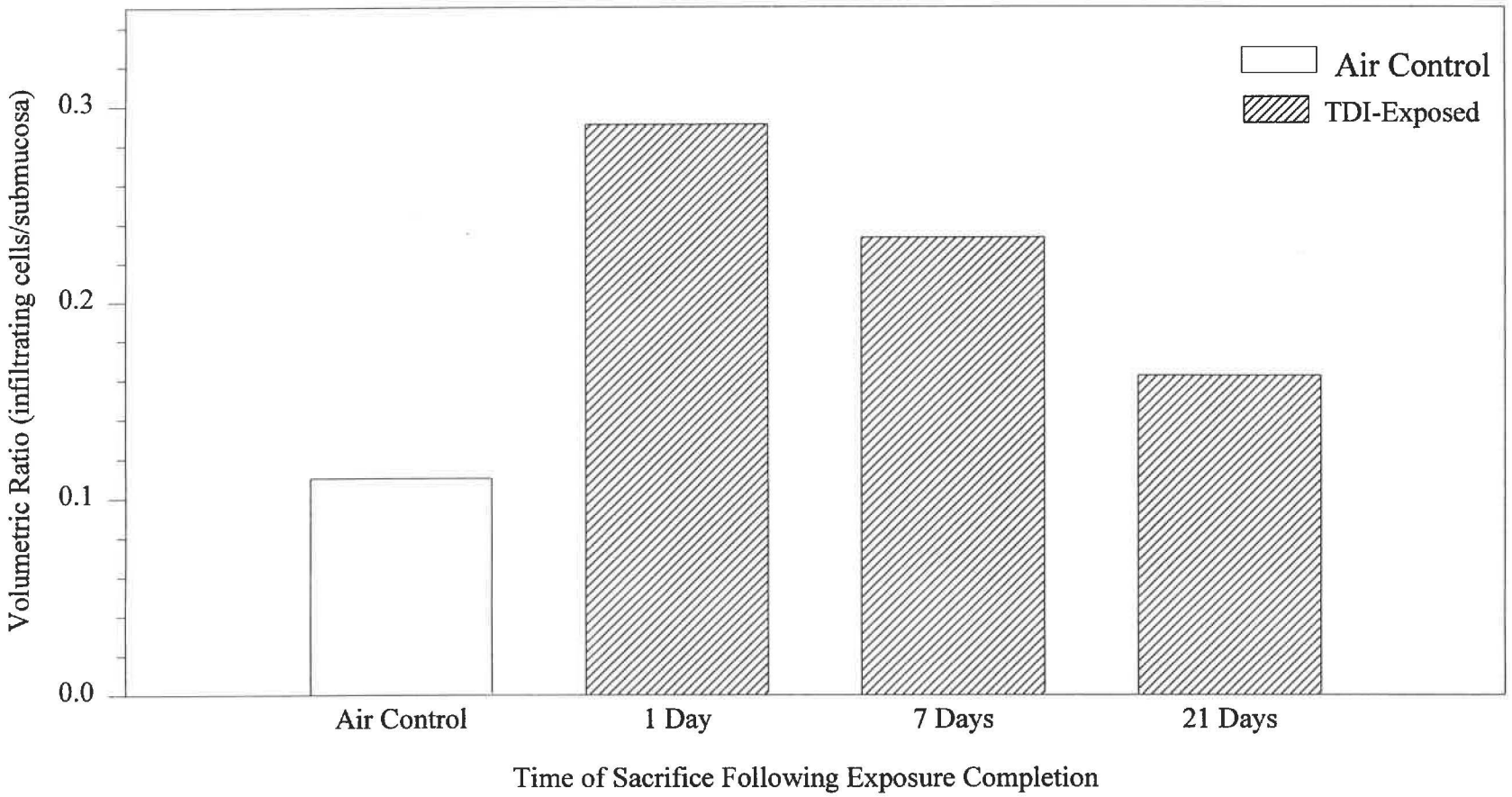
FIGURE 15 - Composite Plate of Representative Photomicrographs Obtained from Air Control (1) and TDI-Exposed Guinea Pig Bronchioles 1 Day (2), 7 Days (3) and 21 Days (4) Post-Exposure Completion.

4.2 Histomorphometric Evaluation

Histomorphometric evaluations were used to provide a semi-quantitative evaluation of the ratio of the volume of infiltrating cells to the submucosal surface area. Following a qualitative evaluation of all slides prepared during the course of these experiments, one control and one TDI-exposed animal displaying representative lesions at each time of sacrifice (1, 7 and 21 days post-exposure completion) were selected for histomorphometric evaluation. Because the slides used for histomorphometric evaluation were selected on the basis that they were representative of the lesions observed at each time point, the data obtained is considered to be indicative of the response observed. This evaluation has been termed "semi-quantitative" as the data presented at each time point was obtained from a single animal. While the evaluation of additional sections would permit statistical evaluation of the results of the histomorphometric evaluation, this additional work will be the focus of future studies. The data obtained are presented graphically in Figure 16 and indicate that 1 day after exposure completion the volume of the submucosa represented by infiltrating cells was approximately 3 times that the control value. Seven and 21 days later, this had further decreased to approximately twice and approximately 1.5 times the control value, respectively.

Figure No. 16

Cellular Infilitration into the Bronchial Submucosa



DISCUSSION

CHAPTER 6

DISCUSSION

The objective of this research was to characterize the effects of TDI vapor exposure in the guinea pig lung, under the conditions established in our laboratory, through the correlation of inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and morphometric effects in the bronchiolar epithelium. This model may be used to examine the mechanisms of TDI-induced occupational asthma. The guinea pig provides a good model for TDI-induced occupational asthma due to numerous similarities with the human situation. These similarities include respiratory tract inflammation, bronchial hyperreactivity to various bronchoconstrictors and similar responses to a wide variety of pharmacological agents including steroids and non-steroidal anti-inflammatory drugs (Cibulas *et al.*, 1986).

The experimental protocol required exposure to TDI vapor at a target concentration of 3 ppm 4 hours/day for 5 consecutive days. Groups of air control and TDI-exposed guinea pigs were sacrificed 1, 7 and 21 days following the completion of exposures to evaluate the effect of TDI vapor on the lung. Investigations conducted in this model were targeted at characterization of two major and clinically relevant features of asthma: the inflammatory response and bronchial hyperreactivity. The inflammatory response was characterized by study of bronchoalveolar lavage fluid and histomorphometric investigation of cellular infiltration into the airways and the bronchial submucosa, respectively. These investigations were supported by qualitative histopathological examinations. Bronchial hyperreactivity was determined using isolated organs techniques to evaluate the responses of the bronchi to non-specific bronchoconstrictors.

The targeted inhalation chamber TDI vapor concentration was 3 ppm. Over the course of the 5 days of exposure, an overall mean chamber concentration of 2.9 ppm ± 0.4 (s.e.m.) was established with individual daily mean chamber concentrations varying between 2.0 ± 0.6 ppm and 3.9 ± 1.9 ppm. Although the daily means were somewhat variable and two individual chamber concentrations differed considerably from the targeted concentration (0.8 ppm and 9.5 ppm obtained during Hour 1 on Exposure Days 4 and 5, respectively), these variations from target were considered not to have affected the results obtained in these experiments. Although 0.02 ppm TDI was apparently measured in the air control atmosphere on Exposure Day 4, this concentration was considered an

artifact resultant from the contamination of one or more of the reagents used in the colorimetric determination of inhalation chamber TDI vapor concentrations. This conclusion was based upon the methods used to contain the exposure and isolate the control group from the TDI-exposed group and was supported by the lack of any colorimetric response in air control samples on any of the 4 other days of TDI exposure nor during the validation of the exposure and generation systems. However, even under the assumption that air control animals were exposed to TDI vapors at a concentration of 0.02 ppm on Exposure Day 4, this exposure would be considered not to have had any impact on the interpretation of the results obtained in this study. During the studies referenced above (Karol, 1983), inhalation exposure to TDI vapors at a concentration of 0.02 ppm for 15 weeks resulted in no detectable dermal or pulmonary sensitivity nor any detectable TDI-specific antibodies. Furthermore, on the basis of previous animal and clinical studies, NIOSH (National Institutes of Occupational Safety and Health, U.S.A.) conservatively established 0.02 ppm as the threshold limit value (TLV) for human exposure to TDI vapors (NIOSH, 1978).

Previous studies (Karol, 1983) have demonstrated a concentration dependence in both the immunologic and physiologic responses to TDI following inhalation exposure. In these studies, guinea pigs exposed to 0.12 ppm TDI for 3 hours/day for 5 days indicated no detectable TDI-specific antibodies in sera and no increase in pulmonary sensitivity as measured by bronchial provocation challenges with a TDI-protein conjugate (TDI-guinea pig serum albumin) but did exhibit evidence of mild sensory irritation. However, exposure at concentrations ranging between 0.36 and 1.0 ppm resulted in TDI-concentration dependant antibody levels, pulmonary sensitivity and sensory irritation (concentration-dependant sensory irritation was established between 0.12 and 0.93 ppm). The dose-response curve indicated a plateau between 1 and 10 ppm providing evidence that the threshold for antibody production was *circa* 1 ppm. Similarly, the proportion of guinea pigs producing antibodies was observed to be concentration dependant: TDI concentrations in excess of 1.0 ppm were demonstrated to result in antibody production in 100% of exposed guinea pigs. In view of the 1 ppm threshold established by Karol and because TDI-induced asthmatic symptoms continue to be considered immunologically-mediated (Mapp, 1994), the exposure concentrations established in this experiment were considered appropriate to meet the objectives of this study.

The exposure to TDI vapor at 3 ppm provided remarkable biological responses. The assessment of airway cell populations by bronchoalveolar lavage (BAL) provided clear evidence of a significant infiltration of inflammatory cells into the airways and the time course of the resolution of this response. Initial statistically significant increases in total numbers of infiltrating cells recovered 1 day after exposure completion returned to control levels 3 weeks later. The infiltration of inflammatory cells into the airways implied by the increases in the total numbers of cells recovered in BAL fluid (BALF) were supported by investigations of the proportion of total infiltrating cells represented by macrophages, eosinophils and neutrophils. Although total numbers of macrophages increased, when expressed as a percentage of total cells, macrophages were initially depressed, returning to control levels 21 days after exposure completion. The macrophage, a phagocytic cell implicated in the defense of the lung predominantly from particulate damage, normally accounts for up to 80-90% of cells retrieved in BALF. Although the absolute numbers of macrophages retrieved in BALF were higher than corresponding controls 1 day after exposure completion, when expressed as a proportion of the total cells recovered in BALF, macrophage populations were statistically significantly lower than control values 1 and 7 days following exposure completion and returned to control levels 3 weeks after exposure completion. These data suggested that the increase in total numbers of cells was not a function of the infiltration of macrophages but was predominantly related to the infiltration of polymorphonuclear leukocytes.

To assess the statistical significance of differences in the proportion of total cells represented by eosinophils and neutrophils, similar analyses were conducted on these cell populations. The expected relationship between the proportion of cells represented by each of these polymorphonuclear leukocytes and the Day of sacrifice (ie. Time*Exposure interaction) was not statistically significant. Therefore, the data collected 1, 7 and 21 days post-exposure were combined and analyzed. The results indicated that both absolute and percentage of total cells represented by eosinophils and neutrophils were increased in TDI-exposed guinea pigs. The proportion of total cells represented by polymorphonuclear leukocytes had returned to normal levels by three weeks post-exposure completion. The neutrophil and eosinophil data was considered supportive of the macrophage data and therefore the BALF data was concluded to provide strong evidence that the increase in total cell numbers recovered in BALF was a result of the infiltration of inflammatory polymorphonuclear leukocytes.

With the exception of recent work by Raulf *et al.* (1995), BALF investigations have not been previously reported for TDI-exposed guinea pigs. In the work reported by Raulf *et al.*, the authors clearly demonstrated eosinophil influx into the airways 1 hour following 2 hour/day exposure to 10, 20 or 30 ppb TDI-vapors for 5 days. While BALF investigations were initiated 1 day post-exposure in our studies, the eosinophil influx observed 1 hour post-exposure by Raulf *et al.* was considered to be consistent with the results obtained in our studies. Though Raulf *et al.* did not observed significant neutrophil infiltration, this may have been a result of the large difference between TDI vapor concentration used by these authors when compared with our studies. Eosinophil infiltration is further supported by BALF analysis in numerous studies conducted in the ovalbumin-sensitized guinea pig, a common model of human asthma in which increased numbers of eosinophils in BALF are considered characteristic of late phase asthmatic response (Mauser *et al.*, 1993). Most importantly, the complete BALF results obtained in our studies are supported by similar observations in numerous clinical studies of occupational exposure to TDI vapors. In these studies both significant eosinophil and neutrophil infiltration were observed (Fabbri, Boschetto and Zocca, 1987).

As the eosinophil infiltration reported by Raulf *et al.* (1995) was consistent with our results, the additional investigations reported by these authors may be relevant to the model described in this research. In their studies, Raulf *et al.* (1995) observed that significantly elevated levels of eosinophils were associated with statistically significant increases in the levels of 5-lipoxygenase-derived lipid mediators LTB_4, and $LTC_4/LTD_4/LTE_4$. In addition, stimulation of BALF cells with calcium ionophore A23187 and arachidonic acid induced increased LTB_4 generation. This last observation was independently supported by work conducted by Mapp *et al.* (1993a) in isolated guinea pig bronchi following TDI-exposure. In these studies, addition of arachidonic acid to the organ bath resulted in the observation of statistically significantly increased LTB_4 levels. These two studies suggest the importance of the 5-lipoxygenase product LTB_4 in the likely cascade which is ultimately manifest as asthma.

Studies of the role of LTB_4 in the propagation of the asthmatic response in the guinea pig do not exist. The data available indicates that the neutrophil may play a key role in TDI-induced and other types of asthma. At present, increased LTB_4 has been clinically correlated with late phase asthmatic response (Zocca *et al.*, 1990). However, as eosinophils and mast cells release LTB_4, and as

neutrophils are an important source of this putative mediator, it is currently unknown whether this leukotriene plays a role in the recruitment of neutrophils or is a consequence of neutrophil infiltration. The guinea pig model characterized in the work presented herein offers the opportunity to study the role of the neutrophil by pharmacologically intervening with specific LTB_4 antagonists. Future studies of pharmacological intervention with LTB_4-specific antagonists may help to elucidate the role of neutrophil-derived LTB_4 in this cascade.

Histopathological evaluation of the sections of the bronchioles and subsequent semi-quantitative histomorphometric evaluation of cellular recruitment into the bronchial submucosa fully supported the BALF results obtained in our experiments. Preliminary qualitative evaluation of all histological sections prepared from the lungs of guinea pigs sacrificed 1, 7 and 21 days after exposure completion clearly indicated epithelial damage. This damage was characterized by epithelial sloughing, replacement of the normal pseudostratified, ciliated columnar epithelium by stratified cuboidal epitheliod cells lacking cilia, clear submucosal inflammation and associated edema (minimal) resultant from the infiltration of polymorphonuclear leukocytes. These observations were most obvious 1 day after the completion of exposure. The beginnings of repair were evident 7 days after exposure completion and although not yet normal, an obvious regeneration of a relatively normal epithelial appearance was evident 21 days after completion of exposure. These observations were generally consistent with the results reported by Miller *et al.* (1986) following 5 days exposure to 3100 ppb TDI and those reported by Cibulas *et al.* (1986) following 5 days of 4 hour/day exposure to 3 ppm TDI.

In their light and electron microscopic studies, Miller *et al.* (1986) observed obvious histopathological changes in the morphology of the bronchial epithelium which was due to TDI exposure 2 and 24 hours after exposure completion. However, these authors did not observe any regenerative changes in the bronchial epithelium 7 days post-exposure. Although our studies indicated the bronchial epithelia had not completely returned to a normal appearance 3 weeks after exposure completion, in view of the minimal differences from the control tissues, the appearance 21 days after exposure was considered consistent with the observation of normal bronchial appearance 3 weeks post-exposure reported by both Miller *et al.* (1986) and Cibulas *et al.* (1986).

Following preliminary microscopic evaluation, lung sections considered representative of the changes observed at each occasion of sacrifice were selected for histomorphometric evaluation. This

evaluation was termed "semi-quantitative" because a single animal was analyzed at each occasion. The data obtained from this histomorphometric evaluation supported the results of BALF analysis. An initial severe inflammatory response characterized by cellular infiltration into the bronchial submucosa was observed which, over the course of 3 weeks, had not fully returned to control levels. One day after exposure completion, the volume of infiltrating cells accounted for approximately 3 times the submucosal surface area observed in the control animal. By seven days after exposure completion, the surface area accounted for by the infiltrating cells had decreased to approximately twice the control submucosal surface area while 3 weeks after exposure completion infiltrating cells accounted for approximately 1.5 times the control values. Although the proportion of the submucosal surface area represented by infiltrating cells observed in the animal sacrificed three weeks after the completion of TDI exposure was higher than the control, the difference in these proportions were considered unlikely to have been important.

Although these histomorphometric data were considered supportive of our BALF data, the limitations of the technique used are important to consider. The point counting technique used in our histomorphometric evaluations may tend to underestimate the infiltration of inflammatory cells into the bronchial submucosa. This method cannot account for swelling of the submucosa due to plasma extravasation (edema), a common observation in asthma. Edema would tend to increase the submucosal surface area without corresponding increases in infiltrating cell numbers or size and therefore tend to result in an underestimation of the submucosal surface area accounted for by the infiltrating cells. However, only minimal edema associated with polymorphonuclear leukocyte infiltration was observed in our studies. Because the histomorphometric data was well supported by the BALF data, the results obtained from the histomorphometric evaluations were considered reliable.

Our BALF and histomorphometric data were considered to be consistent with the results of the morphologic and histomorphometric work reported by Miller *et al.* (1986). Our histomorphometric data was limited to an evaluation of the proportion of the submucosal surface area represented by infiltrating cells (due to the evaluation of a single animal at each occasion of sacrifice, individual cell populations were not evaluated). In contrast, Miller *et al.* (1986) assessed the number of infiltrating leukocytes (polymorphonuclear leukocytes, eosinophils, plasma cells, separately) histomorphometrically 2, 24, 72 hours, 7 and 21 days following 5 days of 4hrs/day 3100 ppb TDI

exposure. The results obtained by these authors indicated that bronchial polymorphonuclear leukocytes attained peak levels 2 hours post-exposure progressively decreasing and returning to control levels by 3 weeks later. Eosinophil numbers were observed to be unchanged from control values 2 and 24 hours post-exposure but were significantly increased from controls 72 hours and 7 days post-exposure with peak levels observed 7 days post-exposure. Three weeks post-exposure, eosinophil levels had fallen but were still high relative to controls.

The lack of significant interaction between Time and Exposure effects precluded the statistical comparison of eosinophil data obtained from BALF analysis 1, 7 and 21 days post-exposure (the data were combined and analyzed together and indicated the eosinophil counts obtained following TDI exposure were significantly higher than corresponding controls). However, with the exception of the higher eosinophil counts 3 weeks post-exposure, the pattern of eosinophil response following completion of TDI exposure was similar to the data reported by Miller *et al.* (1986).

Taken together, these data demonstrate a notable difference in the time course of eosinophil and neutrophil infiltration into the TDI-exposed guinea pig lung. This difference represents a potentially important contrast to the clinical situation and other guinea pig asthma models. Recent studies in the ovalbumin-sensitized guinea pig indicated that 1 day following exposure completion, ovalbumin-induced eosinophilia was much more extensive than observed in our studies representing a 9 fold increase in BALF eosinophil numbers and a 4 to 5 fold increase in intrapulmonary bronchial tissue (Mauser *et al.*, 1993). Studies using monoclonal antibodies directed against adhesion molecules (ICAM-1) have demonstrated delayed or inhibited eosinophil infiltration in the ovalbumin-challenged guinea pig (Ihaku *et al.*, 1994). Though speculative, as the expression of adhesion molecule ICAM-1 appears to be the key factor in the leukocyte endothelial transmigration (Kröegel *et al.*, 1994), it is possible that TDI-exposure affects either the expression of this molecule or the molecule itself following expression, retarding eosinophil infiltration. Because neutrophil infiltration was not similarly affected in either the work reported by Miller *et al.* (1986) or in the work presented herein, the difference in time course of neutrophil and eosinophil infiltration in the TDI-exposed guinea pig suggests neutrophils and eosinophils may be recruited through distinct mechanisms. Further investigations using specific monoclonal antibodies against ICAM-1 and E-selectin, both putative adhesion molecules implicated in leukocyte endothelial transmigration, may

provide additional information to help identify the specific mechanisms of neutrophil infiltration and thereby help elucidate the role of this cell in TDI-induced occupational asthma.

Bronchial hyperreactivity assessments using non-specific agonist challenge of isolated bronchial strips provided clear evidence of a significant increase in bronchial reactivity following TDI-exposure which decreased to control levels within 3 weeks of exposure completion. These experiments were performed using 2 non-specific agonists: histamine, a product released from mast cells following allergen stimulation, and acetylcholine, a neurotransmitter which has been implicated as a trigger in asthmatic episodes. The results obtained with each agonist were virtually identical: initial highly statistically significant increases in bronchial reactivity observed 1 day after exposure completion returned to levels which were statistically similar to controls 1 and 3 weeks following exposure completion. Inspection of the agonist concentration-contractile response curves indicated a stepwise decrease one and three weeks after exposure from the peak contractions observed one day after exposure. These data indicated the time points selected to evaluate bronchial reactivity were appropriate to define the response and supported the results obtained with the other techniques used in our studies (BAL, histopathological evaluation and semi-quantitative histomorphometric evaluation).

The results of our isolated organ experiments support the results of physiological experiments conducted in whole animals by Cibulas *et al.* (1986). In their study, the authors investigated bronchial hyperreactivity in unanesthetized animals by measuring specific airway conductance (SG_{aw}) as a function of increasing doses of aerosolized histamine. The determination of TDI-exposure effect on bronchial hyperreactivity was based on the dose of histamine required to provoke a 50% decrease in SG_{aw} interpolated from the histamine dose vs. SG_{aw} curve (PD_{50} - Provocative Dose, 50%). On the basis of the lack of statistically significant differences between data obtained from air and TDI-exposed animals 3, 7 and 21 days post-exposure completion, the authors determined that the hyperreactivity to non-specific agonist challenge induced by TDI exposure had remitted by 3 days after exposure completion. Although we did not perform any investigations 3 days following exposure completion, these results were similar to those obtained in our study. However, further inspection of the data obtained by Cibulas *et al.* (1986) suggested the lack of statistical significance may have been influenced by the large variability within the SG_{aw}

measurements rather than a lack of difference in bronchial reactivity of TDI-exposed and control animals 7 days after exposure completion (coefficient of variation of 40.1% in control data).

Although the measurement of PD_{50} offers the advantage of an unanesthetized whole animal model, the isolated organ technique used in the present study appears to be a more sensitive indicator of bronchial hyperreactivity.

The data obtained from the bronchial hyperreactivity experiments were consistent with the clear histopathological evidence of epithelial damage observed 1, 7 and 21 days after exposure completion. These data were also consistent with current understandings of the crucial role of the bronchial epithelia in the maintenance of bronchomotor tone. It has been demonstrated that intact epithelial cells produce bronchodilatory endothelium-derived relaxant factor (EDRF), recently identified to be nitric oxide (NO) (Moncada *et al.*, 1991). The loss of the bronchial epithelial cells results in an increase in resting airway smooth muscle tone and an increased sensitivity to various stimuli (Aizawa *et al.*, 1988). These responses have been confirmed in isolated bronchi: studies of the effect of the removal of bronchial epithelia from isolated bronchial strips have demonstrated increased hyperreactivity presumably due to loss of NO and resultant increases in bronchomotor tone (Prie *et al.*, 1990). In addition to the loss of NO, the loss of epithelial cells is thought to result in the exposure of sensory nerves. It has been suggested that subsequent hyperstimulation of these nerves causes bronchoconstriction via reflex neurons (Laitinen, 1985).

The results obtained from BAL, hyperreactivity investigations and histopathological evaluations taken together support current thinking on the development of asthma-like symptoms in this model. Although we observed the percentage of infiltrating cells represented by eosinophils reached a peak 7 days after exposure completion, on the basis of absolute numbers, peak eosinophil levels were observed 1 day post-exposure completion. This was consistent with the clear histopathological evidence of epithelial sloughing and is consistent with the generally accepted theory that eosinophil-derived basic proteins including eosinophil peroxidase (EPO) and eosinophil-derived major basic protein (MBP), are responsible for the epithelial damage which ultimately culminates in the epithelial sloughing observed in the asthmatic lung.

In conclusion, the characterization of the response of the guinea pig lung to TDI vapors described in this research provides an excellent correlation between inflammatory changes in the respiratory tract, bronchial hyperreactivity to agonist challenge, and histopathological and

morphometric changes in the bronchiolar epithelium. This research provides a solid foundation on which to continue investigations into the causes of TDI-induced asthma. This model may be used in the future to study the mechanisms of TDI-induced asthma through pharmacological intervention with the goal of abrogating or inhibiting one or more responses observed in these studies. Primary targets may be the role of leukotriene B_4 in the pathogenesis of asthma.

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APPENDICIES

APPENDIX NO. 1

GROUP MEAN (S.E.M.) BRONCHOALVEOLAR LAVAGE DATA
ABSOLUTE AND DIFFERENTIAL LEUKOCYTE COUNTS

Day	Group No.	Treatment Description	Total Number of Cells Recovered (x1,000,000)	ABSOLUTE CELL COUNTS (x 1,000,000)			DIFFERENTIAL CELL COUNTS		
				Macrophages	Eosinophils	Neutrophils	Macrophages	Eosinophils	Neutrophils
1	1	Air Control	11.82 ± 1.86	10.90 ± 1.97	0.73 ± 0.04	0.19 ± 0.07	91.8% ± 2.17%	6.4% ± 1.38%	1.7% ± 0.87%
	2	TDI-Exposed	51.56 ± 2.77	25.45 ± 2.23	15.97 ± 2.22	10.14 ± 4.49	49.7% ± 4.68%	31.8% ± 4.69%	18.5% ± 7.44%
7	1	Air Control	22.62 ± 1.38	20.54 ± 1.68	1.96 ± 0.27	0.11 ± 0.04	90.7% ± 1.89%	8.7% ± 1.75%	0.5% ± 0.21%
	2	TDI-Exposed	42.02 ± 9.53	24.35 ± 5.54	15.66 ± 3.28	2.03 ± 1.71	58.4% ± 3.16%	38.3% ± 4.28%	3.3% ± 2.37%
21	1	Air Control	21.96 ± 2.04	19.30 ± 3.02	2.58 ± 0.90	0.09 ± 0.09	87.3% ± 5.66%	12.2% ± 5.21%	0.5% ± 0.45%
	2	TDI-Exposed	24.36 ± 2.64	18.56 ± 1.79	5.31 ± 1.19	0.49 ± 0.19	77.1% ± 3.57%	20.8% ± 3.70%	2.0% ± 0.86%

APPENDIX NO. 2

INDIVIDUAL BRONCHOALVEOLAR LAVAGE DATA
ABSOLUTE AND DIFFERENTIAL LEUKOCYTE COUNTS

DAY 1

Group No.	Treatment Description	Animal No.	Total Number of Cells Recovered (x1,000,000)	Absolute Number of Cell Type Recovered (x1,000,000)			Percentage of Total Cells Recovered		
				Macrophages	Eosinophils	Neutrophils	Macrophages	Eosinophils	Neutrophils
1	Air Control	1002	9.96	8.93	0.77	0.26	89.7%	7.7%	2.6%
		1004	13.68	12.86	0.68	0.12	94.0%	5.0%	0.9%
2	TDI-Exposed	2001	47.52	31.70	14.73	1.09	66.7%	31.0%	2.3%
		2002	59.52	28.09	21.37	10.00	47.2%	35.9%	16.8%
		2004	50.40	29.33	16.88	4.28	58.2%	33.5%	8.5%
		2005	46.32	23.53	20.24	2.50	50.8%	43.7%	5.4%
		2006	60.48	23.65	6.05	30.78	39.1%	10.0%	50.9%
		2009	45.12	16.42	16.56	12.18	36.4%	36.7%	27.0%

APPENDIX NO. 2

INDIVIDUAL BRONCHOALVEOLAR LAVAGE DATA
ABSOLUTE AND DIFFERENTIAL LEUKOCYTE COUNTS

DAY 7

Group No.	Treatment Description	Animal No.	Total Number of Cells Recovered (x1,000,000)	Absolute Number of Cell Type Recovered (x1,000,000)			Percentage of Total Cells Recovered		
				Macrophages	Eosinophils	Neutrophils	Macrophages	Eosinophils	Neutrophils
1	Air Control	1007	24.00	22.22	1.68	0.07	92.6%	7.0%	0.3%
		1009	21.24	18.86	2.23	0.15	88.8%	10.5%	0.7%
2	TDI-Exposed	2017	60.00	33.66	25.74	0.60	56.1%	42.9%	1.0%
		2023	30.00	14.94	15.06	0.00	49.8%	50.2%	0.0%
		2024	23.40	16.22	6.81	0.40	69.3%	29.1%	1.7%
		2025	26.88	15.46	11.18	0.27	57.5%	41.6%	1.0%
		2026	69.84	41.48	19.49	8.87	59.4%	27.9%	12.7%

APPENDIX NO. 2

INDIVIDUAL BRONCHOALVEOLAR LAVAGE DATA
ABSOLUTE AND DIFFERENTIAL LEUKOCYTE COUNTS

DAY 21

Group No.	Treatment Description	Animal No.	Total Number of Cells Recovered (x1,000,000)	Absolute Number of Cell Type Recovered (x1,000,000)			Percentage of Total Cells Recovered		
				Macrophages	Eosinophils	Neutrophils	Macrophages	Eosinophils	Neutrophils
1	Air Control	1013	19.92	16.27	3.47	0.18	81.7%	17.4%	0.9%
		1014	24.00	22.32	1.68	0.00	93.0%	7.0%	0.0%
2	TDI-Exposed	2034	18.72	17.17	1.25	0.28	91.7%	6.7%	1.5%
		2038	35.28	26.92	7.94	0.42	76.3%	22.5%	1.2%
		2039	21.84	17.28	3.25	1.31	79.1%	14.9%	6.0%
		2040	24.00	16.30	7.44	0.24	67.9%	31.0%	1.0%
		2041	18.24	14.46	3.78	0.00	79.3%	20.7%	0.0%
		2045	28.08	19.21	8.17	0.70	68.4%	29.1%	2.5%

APPENDIX NO. 3

GROUP MEAN (S.E.M.) BRONCHIAL HYPERREACTIVITY DATA
HISTAMINE CHALLENGE

TOTAL RESPONSE

CONTRACTILE FORCE (g)

Histamine Concentration		DAY 1		DAY 7		DAY 21	
(μg)	(-log mol)	Air Control	TDI-Exposed	Air Control	TDI-Exposed	Air Control	TDI-Exposed
0.1	9.05	0.003 ± 0.003	0.024 ± 0.006	0.000 ± 0.000	0.002 ± 0.001	0.007 ± 0.006	0.008 ± 0.003
0.5	8.35	0.015 ± 0.006	0.128 ± 0.031	0.013 ± 0.004	0.032 ± 0.004	0.016 ± 0.009	0.035 ± 0.010
1	8.05	0.025 ± 0.009	0.242 ± 0.069	0.055 ± 0.025	0.048 ± 0.006	0.026 ± 0.007	0.056 ± 0.015
2	7.74	0.053 ± 0.030	0.451 ± 0.094	0.029 ± 0.012	0.079 ± 0.014	0.040 ± 0.015	0.103 ± 0.034
5	7.35	0.095 ± 0.044	0.910 ± 0.130	0.049 ± 0.010	0.150 ± 0.028	0.065 ± 0.016	0.180 ± 0.056
10	7.05	0.186 ± 0.117	1.163 ± 0.126	0.064 ± 0.001	0.237 ± 0.055	0.077 ± 0.021	0.275 ± 0.083
20	6.74	0.252 ± 0.149	1.381 ± 0.132	0.079 ± 0.010	0.354 ± 0.069	0.122 ± 0.021	0.364 ± 0.111
50	6.35	0.385 ± 0.235	1.614 ± 0.139	0.108 ± 0.026	0.547 ± 0.073	0.164 ± 0.035	0.506 ± 0.142
100	6.05	0.418 ± 0.256	1.467 ± 0.164	0.110 ± 0.022	0.535 ± 0.057	0.187 ± 0.046	0.561 ± 0.148
200	5.74	0.413 ± 0.225	1.363 ± 0.147	0.116 ± 0.032	0.503 ± 0.035	0.176 ± 0.044	0.563 ± 0.155
AUC* (μg·g)		84.5 ± 43.9	283.9 ± 27.4	29.1 ± 4.5	96.7 ± 10.8	46.3 ± 7.7	100.8 ± 27.6

* AUC = area under the acetylcholine dose (μg) * contractile force (g) response curve.

APPENDIX NO. 4

INDIVIDUAL BRONCHIAL HYPERREACTIVITY DATA
HISTAMINE CHALLENGE
DAY 1

CONTRACTILE FORCE (g)

Histamine Concentration		Group 1: Air Control						Group 2: TDI - Exposed											
		1001			1003			2003			2007			2008			2010		
		Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total
(µg)	(-log mol)	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force
0.1	9.05	0.000	0.000	0.000	0.000	0.006	0.006	0.010	0.014	0.024	0.018	0.023	0.041	0.000	0.010	0.010	0.011	0.011	0.022
0.5	8.35	0.004	0.009	0.013	0.010	0.014	0.024	0.073	0.035	0.108	0.104	0.113	0.217	0.038	0.034	0.072	0.059	0.056	0.115
1	8.05	0.011	0.010	0.021	0.018	0.021	0.039	0.150	0.054	0.204	0.223	0.218	0.441	0.060	0.058	0.118	0.100	0.105	0.205
2	7.74	0.014	0.016	0.030	0.049	0.041	0.090	0.265	0.093	0.358	0.350	0.370	0.720	0.168	0.125	0.293	0.218	0.215	0.433
5	7.35	0.030	0.039	0.069	0.081	0.075	0.156	0.488	0.203	0.691	0.563	0.663	1.226	0.440	0.265	0.705	0.555	0.463	1.018
10	7.05	0.044	0.048	0.092	0.213	0.113	0.326	0.638	0.260	0.898	0.688	0.725	1.413	0.638	0.363	1.001	0.763	0.575	1.338
20	6.74	0.075	0.063	0.138	0.260	0.175	0.435	0.738	0.288	1.026	0.725	0.725	1.450	0.813	0.573	1.386	0.975	0.688	1.663
50	6.35	0.128	0.073	0.201	0.395	0.275	0.670	0.825	0.413	1.238	0.800	0.775	1.575	1.130	0.663	1.793	1.100	0.750	1.850
100	6.05	0.153	0.063	0.216	0.413	0.315	0.728	0.700	0.388	1.088	0.675	0.625	1.300	1.080	0.688	1.768	1.075	0.638	1.713
200	5.74	0.175	0.075	0.250	0.400	0.300	0.700	0.675	0.375	1.050	0.650	0.525	1.175	1.063	0.563	1.626	1.025	0.575	1.600
AUC* (µg·g)		27.3	13.2	40.5	74.0	54.4	128.4	141.4	73.1	214.6	138.0	127.7	265.6	202.6	121.8	324.4	203.9	127.1	330.9

* AUC = area under the histamine dose (µg) * contractile force (g) response curve.

APPENDIX NO. 4

INDIVIDUAL BRONCHIAL HYPERREACTIVITY DATA
HISTAMINE CHALLENGE
DAY 7

CONTRACTILE FORCE (g)

Histamine Concentration		Group 1: Air Control						Group 2: TDI - Exposed											
		1001			1003			2003			2007			2008			2010		
		Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total
(µg)	(-log mol)	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force
0.1	9.05	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.004	0.000	0.000	0.000	0.003	0.000	0.003	0.000	0.000	0.000
0.5	8.35	0.009	0.006	0.015	0.009	0.014	0.023	0.019	0.025	0.044	0.013	0.013	0.026	0.014	0.011	0.025	0.010	0.023	0.033
1	8.05	0.080	0.010	0.090	0.015	0.025	0.040	0.025	0.041	0.066	0.018	0.028	0.046	0.023	0.019	0.042	0.013	0.026	0.039
2	7.74	0.013	0.018	0.031	0.020	0.035	0.055	0.045	0.076	0.121	0.025	0.039	0.064	0.034	0.026	0.060	0.025	0.045	0.070
5	7.35	0.026	0.033	0.059	0.030	0.049	0.079	0.098	0.133	0.231	0.054	0.060	0.114	0.065	0.046	0.111	0.068	0.075	0.143
10	7.05	0.038	0.046	0.084	0.033	0.053	0.086	0.145	0.245	0.390	0.080	0.070	0.150	0.108	0.054	0.162	0.128	0.118	0.246
20	6.74	0.050	0.049	0.099	0.043	0.076	0.119	0.210	0.325	0.535	0.145	0.093	0.238	0.163	0.093	0.256	0.220	0.165	0.385
50	6.35	0.075	0.051	0.126	0.053	0.125	0.178	0.330	0.413	0.743	0.255	0.153	0.408	0.280	0.190	0.470	0.335	0.230	0.565
100	6.05	0.079	0.053	0.132	0.061	0.115	0.176	0.315	0.375	0.690	0.280	0.143	0.423	0.285	0.200	0.485	0.315	0.225	0.540
200	5.74	0.080	0.054	0.134	0.069	0.128	0.197	0.295	0.300	0.595	0.285	0.140	0.425	0.295	0.195	0.490	0.305	0.195	0.500
AUC* (µg·g)		14.4	10.2	24.6	11.4	22.2	33.7	57.4	68.7	126.1	49.2	26.6	75.8	51.7	34.9	86.6	58.0	40.4	98.4

* AUC = area under the histamine dose (µg) * contractile force (g) response curve.

APPENDIX NO. 4

INDIVIDUAL BRONCHIAL HYPERREACTIVITY DATA
HISTAMINE CHALLENGE
DAY 21

CONTRACTILE FORCE (g)

Histamine Concentration		Group 1: Air Control						Group 2: TDI - Exposed											
		1001			1003			2003			2007			2008			2010		
		Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total
(μg)	(-log mol)	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force
0.1	9.05	0.000	0.005	0.005	0.006	0.011	0.017	0.000	0.001	0.001	0.009	0.008	0.017	0.000	0.004	0.004	0.003	0.005	0.008
0.5	8.35	0.000	0.016	0.016	0.019	0.014	0.033	0.000	0.011	0.011	0.025	0.014	0.039	0.023	0.035	0.058	0.010	0.020	0.030
1	8.05	0.011	0.018	0.029	0.021	0.023	0.044	0.009	0.015	0.024	0.029	0.024	0.053	0.029	0.069	0.098	0.020	0.030	0.050
2	7.74	0.023	0.020	0.043	0.046	0.026	0.072	0.015	0.018	0.033	0.050	0.033	0.083	0.056	0.138	0.194	0.044	0.058	0.102
5	7.35	0.048	0.028	0.076	0.073	0.035	0.108	0.039	0.028	0.067	0.089	0.048	0.137	0.095	0.235	0.330	0.095	0.090	0.185
10	7.05	0.058	0.031	0.089	0.090	0.041	0.131	0.054	0.036	0.090	0.118	0.076	0.194	0.138	0.325	0.463	0.173	0.178	0.351
20	6.74	0.108	0.041	0.149	0.145	0.046	0.191	0.068	0.050	0.118	0.170	0.100	0.270	0.233	0.400	0.633	0.250	0.185	0.435
50	6.35	0.150	0.045	0.195	0.205	0.059	0.264	0.135	0.066	0.201	0.200	0.163	0.363	0.350	0.500	0.850	0.375	0.235	0.610
100	6.05	0.168	0.051	0.219	0.245	0.066	0.311	0.180	0.086	0.266	0.195	0.180	0.375	0.363	0.550	0.913	0.425	0.265	0.690
200	5.74	0.155	0.050	0.205	0.235	0.059	0.294	0.200	0.095	0.295	0.185	0.155	0.340	0.388	0.573	0.961	0.400	0.255	0.655
AUC* (μg·g)		29.2	9.4	38.5	42.3	11.7	54.0	30.9	15.3	46.1	36.7	30.6	67.3	66.8	101.6	168.5	73.7	47.6	121.2

* AUC = area under the histamine dose (μg) * contractile force (g) response curve.

APPENDIX NO. 5

GROUP MEAN (S.E.M.) BRONCHIAL HYPERREACTIVITY DATA
ACETYLCHOLINE CHALLENGE

TOTAL RESPONSE

CONTRACTILE FORCE (g)

Acetylcholine Concentration		DAY 1		DAY 7		DAY 21	
(µg)	(-log mol)	Air Control	TDI-Exposed	Air Control	TDI-Exposed	Air Control	TDI-Exposed
0.1	9.12	0.002 ± 0.002	0.007 ± 0.002	0.000 ± 0.000	0.007 ± 0.001	0.002 ± 0.001	0.008 ± 0.004
0.5	8.42	0.011 ± 0.005	0.039 ± 0.005	0.002 ± 0.003	0.019 ± 0.002	0.009 ± 0.003	0.034 ± 0.012
1	8.12	0.020 ± 0.008	0.064 ± 0.012	0.006 ± 0.003	0.032 ± 0.005	0.011 ± 0.003	0.049 ± 0.017
2	7.82	0.035 ± 0.011	0.107 ± 0.018	0.012 ± 0.004	0.046 ± 0.008	0.015 ± 0.004	0.078 ± 0.030
5	7.42	0.070 ± 0.021	0.201 ± 0.036	0.017 ± 0.005	0.077 ± 0.013	0.029 ± 0.010	0.128 ± 0.049
10	7.12	0.097 ± 0.032	0.289 ± 0.049	0.027 ± 0.007	0.112 ± 0.021	0.042 ± 0.013	0.168 ± 0.064
20	6.82	0.174 ± 0.067	0.431 ± 0.066	0.038 ± 0.012	0.155 ± 0.023	0.058 ± 0.020	0.227 ± 0.082
50	6.42	0.238 ± 0.090	0.696 ± 0.104	0.050 ± 0.018	0.232 ± 0.034	0.085 ± 0.029	0.295 ± 0.100
100	6.12	0.247 ± 0.092	0.823 ± 0.115	0.054 ± 0.019	0.280 ± 0.046	0.105 ± 0.035	0.355 ± 0.119
200	5.82	0.253 ± 0.087	0.989 ± 0.123	0.054 ± 0.023	0.311 ± 0.059	0.111 ± 0.036	0.378 ± 0.126
500	5.42	0.255 ± 0.089	1.131 ± 0.118	0.062 ± 0.033	0.387 ± 0.076	0.122 ± 0.043	0.419 ± 0.138
1000	5.12	0.259 ± 0.092	1.210 ± 0.119	0.067 ± 0.039	0.420 ± 0.074	0.135 ± 0.044	0.467 ± 0.144
1500	4.94	0.232 ± 0.076	1.148 ± 0.110	0.059 ± 0.035	0.378 ± 0.073	0.134 ± 0.046	0.458 ± 0.139
AUC* (µg·g)		426.9 ± 209.4	1644 ± 172.4	137.3 ± 48.7	555.6 ± 101.9	250.5 ± 12.5	636.0 ± 201.6

* AUC = area under the acetylcholine dose (µg) * contractile force (g) response curve.

APPENDIX NO. 6

INDIVIDUAL BRONCHIAL HYPERREACTIVITY DATA
ACETYLCHOLINE CHALLENGE
DAY 1

CONTRACTILE FORCE (g)

Acetylcholine Concentration		Group 1: Air Control						Group 2: TDI - Exposed											
		1001			1003			2003			2007			2008			2010		
		Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total
(µg)	(-log mol)	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force
0.1	9.12	0.000	0.000	0.000	0.004	0.000	0.004	0.001	0.003	0.004	0.009	0.003	0.012	0.005	0.000	0.005	0.000	0.008	0.008
0.5	8.42	0.003	0.001	0.004	0.011	0.009	0.020	0.018	0.010	0.028	0.023	0.016	0.039	0.019	0.016	0.035	0.028	0.024	0.052
1	8.12	0.004	0.006	0.010	0.019	0.016	0.035	0.024	0.021	0.045	0.033	0.036	0.069	0.029	0.018	0.047	0.046	0.049	0.095
2	7.82	0.013	0.011	0.024	0.029	0.028	0.057	0.049	0.033	0.082	0.053	0.051	0.104	0.055	0.030	0.085	0.080	0.078	0.158
5	7.42	0.025	0.025	0.050	0.059	0.055	0.114	0.090	0.055	0.145	0.088	0.075	0.163	0.133	0.057	0.190	0.160	0.145	0.305
10	7.12	0.041	0.021	0.062	0.079	0.083	0.162	0.155	0.070	0.225	0.128	0.110	0.238	0.170	0.088	0.258	0.215	0.220	0.435
20	6.82	0.061	0.029	0.090	0.185	0.118	0.303	0.230	0.108	0.338	0.170	0.153	0.323	0.300	0.153	0.453	0.305	0.305	0.610
50	6.42	0.086	0.041	0.127	0.250	0.163	0.413	0.365	0.200	0.565	0.255	0.230	0.485	0.525	0.270	0.795	0.538	0.400	0.938
100	6.12	0.098	0.041	0.139	0.260	0.165	0.425	0.413	0.255	0.668	0.315	0.270	0.585	0.638	0.350	0.988	0.600	0.450	1.050
200	5.82	0.113	0.040	0.153	0.240	0.190	0.430	0.563	0.305	0.868	0.363	0.338	0.701	0.763	0.400	1.163	0.650	0.573	1.223
500	5.42	0.118	0.030	0.148	0.240	0.195	0.435	0.638	0.385	1.023	0.463	0.388	0.851	0.888	0.438	1.326	0.775	0.550	1.325
1000	5.12	0.115	0.031	0.146	0.235	0.210	0.445	0.663	0.405	1.068	0.573	0.375	0.948	0.900	0.525	1.425	0.800	0.600	1.400
1500	4.94	0.103	0.044	0.147	0.195	0.195	0.390	0.588	0.390	0.978	0.573	0.363	0.936	0.850	0.488	1.338	0.788	0.550	1.338
AUC* (µg·g)		165.5	52.1	217.6	344.4	291.9	636.3	898.1	545.1	1443	726.2	534.8	1261	1247	680.8	1928	1112	830.4	1942

* AUC = area under the acetylcholine dose (µg) * contractile force (g) response curve.

APPENDIX NO. 6

INDIVIDUAL BRONCHIAL HYPERREACTIVITY DATA
ACETYLCHOLINE CHALLENGE
DAY 7

CONTRACTILE FORCE (g)

Acetylcholine Concentration		Group 1: Air Control						Group 2: TDI - Exposed											
		1001			1003			2003			2007			2008			2010		
		Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total
(µg)	(-log mol)	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force
0.1	9.12	0.000	0.000	0.000	0.000	0.001	0.001	0.004	0.005	0.009	0.004	0.005	0.009	0.001	0.003	0.004	0.000	0.004	0.004
0.5	8.42	0.000	0.000	0.000	0.000	0.008	0.008	0.009	0.015	0.024	0.006	0.015	0.021	0.003	0.011	0.014	0.004	0.013	0.017
1	8.12	0.000	0.005	0.005	0.004	0.008	0.012	0.019	0.026	0.045	0.011	0.019	0.030	0.008	0.013	0.021	0.018	0.014	0.032
2	7.82	0.008	0.006	0.014	0.008	0.010	0.018	0.033	0.034	0.067	0.020	0.016	0.036	0.013	0.019	0.032	0.023	0.026	0.049
5	7.42	0.011	0.011	0.022	0.010	0.015	0.025	0.054	0.055	0.109	0.031	0.034	0.065	0.024	0.025	0.049	0.045	0.040	0.085
10	7.12	0.023	0.015	0.038	0.014	0.018	0.032	0.080	0.083	0.163	0.048	0.041	0.089	0.045	0.025	0.070	0.078	0.048	0.126
20	6.82	0.030	0.018	0.048	0.023	0.031	0.054	0.113	0.105	0.218	0.083	0.056	0.139	0.066	0.041	0.107	0.095	0.060	0.155
50	6.42	0.040	0.021	0.061	0.033	0.045	0.078	0.170	0.153	0.323	0.123	0.068	0.191	0.108	0.060	0.168	0.155	0.089	0.244
100	6.12	0.041	0.024	0.065	0.039	0.046	0.085	0.205	0.205	0.410	0.145	0.070	0.215	0.138	0.080	0.218	0.165	0.113	0.278
200	5.82	0.039	0.021	0.060	0.038	0.059	0.097	0.230	0.255	0.485	0.155	0.070	0.225	0.170	0.088	0.258	0.170	0.105	0.275
500	5.42	0.041	0.019	0.060	0.041	0.085	0.126	0.285	0.320	0.605	0.180	0.078	0.258	0.215	0.113	0.328	0.205	0.150	0.355
1000	5.12	0.046	0.016	0.062	0.044	0.101	0.145	0.310	0.315	0.625	0.190	0.083	0.273	0.255	0.130	0.385	0.225	0.170	0.395
1500	4.94	0.033	0.019	0.052	0.038	0.093	0.131	0.265	0.315	0.580	0.165	0.068	0.233	0.250	0.118	0.368	0.170	0.160	0.330
AUC* (µg·g)		61.0	27.7	88.7	60.4	125.6	186.0	406.6	439.8	846.4	257.2	113.3	370.5	326.5	166.9	493.3	292.3	219.8	512.1

* AUC = area under the acetylcholine dose (µg) * contractile force (g) response curve.

APPENDIX NO. 6

INDIVIDUAL BRONCHIAL HYPERREACTIVITY DATA
ACETYLCHOLINE CHALLENGE
DAY 21

CONTRACTILE FORCE (g)

Acetylcholine Concentration		Group 1: Air Control						Group 2: TDI - Exposed											
		1001			1003			2003			2007			2008			2010		
		Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total	Bronchus		Total
(µg)	(-log mol)	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force	Left	Right	Force
0.1	9.12	0.000	0.003	0.003	0.003	0.000	0.003	0.000	0.000	0.000	0.009	0.004	0.013	0.010	0.006	0.016	0.004	0.000	0.004
0.5	8.42	0.008	0.004	0.012	0.008	0.004	0.012	0.000	0.004	0.004	0.014	0.010	0.024	0.024	0.031	0.055	0.034	0.018	0.052
1	8.12	0.010	0.006	0.016	0.008	0.005	0.013	0.005	0.008	0.013	0.015	0.014	0.029	0.024	0.050	0.074	0.049	0.031	0.080
2	7.82	0.013	0.009	0.022	0.010	0.006	0.016	0.005	0.010	0.015	0.021	0.018	0.039	0.041	0.080	0.121	0.083	0.054	0.137
5	7.42	0.033	0.010	0.043	0.019	0.009	0.028	0.014	0.013	0.027	0.033	0.029	0.062	0.071	0.128	0.199	0.145	0.078	0.223
10	7.12	0.045	0.015	0.060	0.034	0.013	0.047	0.025	0.016	0.041	0.041	0.035	0.076	0.090	0.175	0.265	0.175	0.113	0.288
20	6.82	0.063	0.016	0.079	0.046	0.026	0.072	0.031	0.021	0.052	0.059	0.064	0.123	0.128	0.260	0.388	0.205	0.140	0.345
50	6.42	0.093	0.025	0.118	0.076	0.026	0.102	0.068	0.029	0.097	0.079	0.073	0.152	0.190	0.305	0.495	0.270	0.165	0.435
100	6.12	0.110	0.034	0.144	0.100	0.031	0.131	0.088	0.039	0.127	0.098	0.085	0.183	0.235	0.385	0.620	0.300	0.190	0.490
200	5.82	0.108	0.040	0.148	0.118	0.031	0.149	0.103	0.048	0.151	0.108	0.088	0.196	0.275	0.413	0.688	0.285	0.190	0.475
500	5.42	0.108	0.044	0.152	0.145	0.039	0.184	0.135	0.061	0.196	0.133	0.088	0.221	0.340	0.450	0.790	0.275	0.195	0.470
1000	5.12	0.125	0.050	0.175	0.145	0.044	0.189	0.158	0.079	0.237	0.138	0.098	0.236	0.360	0.475	0.835	0.345	0.215	0.560
1500	4.94	0.120	0.049	0.169	0.158	0.039	0.197	0.178	0.081	0.259	0.145	0.093	0.238	0.370	0.463	0.833	0.295	0.205	0.500
AUC* (µg·g)		171.0	66.9	237.9	205.4	57.6	263.0	208.3	98.5	306.8	192.2	136.1	328.3	492.4	664.2	1157	452.8	299.7	752.5

* AUC = area under the acetylcholine dose (µg) * contractile force (g) response curve.