THE DEVELOPMENT AND EVALUATION OF A NEW AEROSOL IRRITANT ASSAY
WITH MINIMAL ANIMAL STRESS

by

ANDRZEJ STANISLAW KARWOWSKI

B.Sc., The University of British Columbia, 1998

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pharmacology & Therapeutics, Faculty of Medicine)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2000

© Andrzej S. Karwowski, 2000

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pharmacology and Therapeutics.

The University of British Columbia
Vancouver, Canada

Date April 20 2000

ABSTRACT

Current methods evaluate pulmonary irritants by placing experimental animals in closed chambers and exposing them to fixed concentrations of irritants for fixed periods of time. This unfortunately results in significant amounts of physical and psychological distress for the animals since they are trapped in the noxious environment without any possibility of escape. The objective of this project was to develop a new assay to evaluate pulmonary irritants in mice, while minimising the stress experienced by the animals. Thus, this assay was based on the principle of avoidance.

The Minimal Animal Stress Irritant Assay Chamber (MASIAC) consists of two symmetrical chambers separated by a central disc with an opening, through which the mice can move freely. An irritant aerosol is introduced into one chamber and a control (non-irritating) aerosol into the other. If the animals feel any irritation or discomfort because of the irritant aerosol, they have the opportunity to escape the environment as soon as they detect it.

The MASIAC was evaluated and the results indicate that it is an effective, reproducible and sensitive assay. Analysing the EC50s of 112 mice, they were found to be $\log_{10}$ -normally distributed, with a mean of -1.57 and standard deviation of 0.48.

The irritation that the MASIAC detects is not limited to any particular type of irritant, because it has the potential to detect any form of discomfort that the mice perceive.

Since a group of mice were tested at the same time, the influence of the group on the responses of the mice was investigated. It was found that the mice tended to avoid clustering together.

When multiple experiments were performed on the same group of animals, it was found that the mice were either sensitised to the irritant, or they learned to avoid it.

There are a number of possible avenues for future research for the MASIAC, including environmental pollution, human asthma, and as a quantifiable model of learning.

TABLE OF CONTENTS

ABSTRACT	ii
TABLE OF CONTENTS	iv
LIST OF TABLES.....	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS.....	x
ACKNOWLEDGMENTS	xi
1.INTRODUCTION	1
1.1. Pulmonary Irritation	1
1.1.1. Definitions	1
1.2. Anatomy and Physiology of the Pulmonary System	1
1.2.1. Rapidly Adapting Receptors.....	2
1.2.2. C Fibre Receptors	2
1.2.3. Other Receptor Subtypes.....	3
1.3. Methods of Evaluating Pulmonary Irritation	3
1.3.1. Animal Body Plethysmograph	3
1.3.2. Measuring Cough	4
1.3.3. Biochemical Methods of Evaluating Irritants.....	5
1.4. Exposure Systems	5
1.4.1. Static Exposure Systems	6
1.4.2. Recirculating Exposure Systems.....	6
1.4.3. Dynamic Exposure Systems.....	7
1.4.3.1. Head-Only Exposure Chamber	7
1.4.3.2. Mouth/Nose-Only Exposure Chamber	8
1.4.3.3. Partial Lung/Lung-Only Exposure Chamber	8
1.4.3.4. Whole-Body Exposure Chamber	9
1.5. Factors to Consider with Exposure Systems	10
1.5.1. Temperature.....	10
1.5.2. Relative Humidity	10
1.5.3. Uniform Distribution.....	11

1.5.4.	Deposition of the Test Substance on the Animal's Fur	11
1.5.5.	Types of Test Substances.....	11
1.5.5.1.	Ultrasonic Nebulizers	12
1.5.5.2.	Jet Nebulizers.....	12
1.5.5.3.	Other Methods	13
1.5.5.4.	Aerosol Dosimetry.....	13
1.6.	Rationale for the Study	14
2.	MATERIALS & METHODS.....	16
2.1.	Apparatus.....	16
2.2.	Animals and Drugs.....	16
2.3.	Method I.....	18
2.4.	Method II.....	19
2.4.1.	Justification for Time Points	19
2.4.2.	Calculation of Percent Irritation	20
2.4.3.	Calculation of EC50.....	21
2.5.	Modifying the MASIAC Chamber.....	21
2.6.	Method III.....	22
2.6.1.	Calculation of EC50.....	23
3.	RESULTS.....	24
3.1.	Method I.....	24
3.1.1.	Response to 1.4 M Citric Acid	24
3.1.2.	Absence of Desensitisation	24
3.2.	Method II.....	27
3.2.1.	MASIAC Control Experiments	28
3.2.2.	Citric Acid and Capsaicin Dose-Response Curves	28
3.3.	Method III.....	30
3.3.1.	Dose-Response Curves to Four Pulmonary Irritants	30
3.3.1.1.	Distribution of Citric Acid EC50 Values	33
3.3.2.	Group Effect	33
3.3.3.	Modification of the Dose-Response Curves by Morphine and	

3.3.4.	Capsazepine	42
3.3.4.	Reproducibility of the Results	52
3.3.5.	Absence of Desensitisation	54
4.	DISCUSSION	60
4.1.	MASIAC Chamber	60
4.1.1.	Factors to Consider with Exposure Systems	60
4.1.1.1.	Temperature and Humidity	60
4.1.1.2.	Uniform Distribution and Aerosol Deposition	61
4.1.1.3.	Type of Nebulizer	61
4.1.2.	Evaluation of a New Assay	61
4.1.2.1.	Accuracy	62
4.1.2.2.	Precision	62
4.1.2.3.	Sensitivity	63
4.1.2.4.	Specificity	64
4.1.2.5.	Incidence of False Positive/Negative Results	64
4.1.2.6.	Cost and Time Required	65
4.1.2.7.	Testing Groups of Mice Simultaneously	65
4.2.	Pharmacology of the Respiratory Tract	66
4.2.1.	Citric Acid and Capsaicin	66
4.2.2.	Sodium Metabisulfite	67
4.2.3.	Ammonia	68
4.3.	Future Avenues for Research	69
4.3.1.	Atmospheric and Environmental Sciences	69
4.3.2.	Animal Behavioural Research	69
4.3.3.	Asthma	69
4.4.	Conclusion	70
5.	BIBLIOGRAPHY	71

LIST OF TABLES

Table 1:	Results from One 5-minute Experimental Run with a Citric Acid Concentration of 2.4 mM.	40
Table 2:	Averaged Correlation R-Values from 50 Mice under Different Experimental Conditions.	41
Table 3:	EC50s of Four Pulmonary Irritants, and their Modification by Morphine and Capsazepine.	51
Table 4:	Differences in EC50s to Four Pulmonary Irritants with Either Half or All the Mice Without an Antagonist.	53

LIST OF FIGURES

Figure 1:	(a) Schematic Diagram of the Minimal Animal Stress Irritant Assay Chamber (MASIAC); (b) Picture of the MASIAC with Mice.	17
Figure 2:	Exposure of Six Mice to an Aerosol of Distilled Water and a Single Concentration of Citric Acid.	25
Figure 3:	Multiple Testing with 1.4 M Citric Acid.	26
Figure 4:	MASIAC Control Runs.	29
Figure 5:	Citric Acid Dose-Response Curve.	31
Figure 6:	Capsaicin Dose-Response Curve.	32
Figure 7:	Citric Acid Dose-Response Curve.	34
Figure 8:	Capsaicin Dose-Response Curve.	35
Figure 9:	Sodium Metabisulfite Dose-Response Curve.	36
Figure 10:	Ammonia Dose-Response Curve.	37
Figure 11:	Cumulative Distribution Histograms of Citric Acid EC50 Values.	38
Figure 12:	Citric Acid Dose-Response Curves with and without 10 mg/kg Morphine.	43
Figure 13:	Citric Acid Dose-Response Curves with and without 18.8 mg/kg Capsazepine.	44
Figure 14:	Capsaicin Dose-Response Curves with and without 10 mg/kg Morphine.	45
Figure 15:	Capsaicin Dose-Response Curves with and without 18.8 mg/kg Capsazepine.	46
Figure 16:	Sodium Metabisulfite Dose-Response Curves with and without 10 mg/kg Morphine.	47
Figure 17:	Sodium Metabisulfite Dose-Response Curves with and without 18.8 mg/kg Capsazepine.	48

Figure 18:	Ammonia Dose-Response Curves with and without 10 mg/kg Morphine.	49
Figure 19:	Ammonia Dose-Response Curves with and without 18.8 mg/kg Capsazepine.	50
Figure 20:	Four Exposures of Citric Acid Dose-Response Curves on the Same Group of Mice.	55
Figure 21:	Four Exposures of Capsaicin Dose-Response Curves on the Same Group of Mice.	56
Figure 22:	Four Exposures of Sodium Metabisulfite Dose-Response Curves on the Same Group of Mice.	57
Figure 23:	Four Exposures of Ammonia Dose-Response Curves on the Same Group of Mice.	58

LIST OF ABBREVIATIONS

BC	British Columbia
EC50	50% Effective Concentration
g	grams
ip	intraperitoneal
iv	intravenous
L	litres
L/min	litres per minute time
M	molar
MASIAC	Minimal Animal Stress Irritant Assay Chamber
MMAD	Mass Median Aerodynamic Diameter
µm	micrometres
µM	micromolar
µmol/kg	micromoles per kilogram weight
mg	milligrams
mg/kg	milligrams per kilogram weight
mg/ml	milligrams per millilitre volume
ml	millilitre
mM	millimolar
n=	sample size
Na	sodium
p	probability
RD50	the concentration of irritant needed to decrease the respiratory rate by 50%
sc	subcutaneous
SEM	Standard Error of the Mean
TCAS	Test Chamber Avoidance Score
UBC	University of British Columbia
UK	United Kingdom

ACKNOWLEDGMENTS

First and foremost, I would like to thank my family and friends, including my parents, my brother Peter, Ms. Silver Anderson, Mr. Andy Laycock, Ms. Sonia Franciosi and Mr. Daniel Leung, for all their support and assistance over the last few years, as well as their patience and understanding as to why I had to spend 12-hour days and weekends in the laboratory. I will never forget all your words of encouragement and motivation.

I would also like to acknowledge all the members of the Clinical Pharmacology Research Organisation, especially Mr. Noam Butterfield, Mr. Lui Franciosi, Mr. Edgar Jimenez, Ms. Auralyn MacKenzie, Mr. Clifford Pau and Mr. Robin Yu, for all their advice, numerous discussions, enthusiasm and helpful suggestions.

I would like to thank all the members and staff of the Department of Pharmacology & Therapeutics for all their help with regards to the departmental paperwork, especially Mr. Christian Caritey, Mrs. Wynne Leung, Mrs. Bick Lu, Mrs. Maureen Murphy, and Mrs. Janelle Stewart. In addition, I would like to recognise Dr. Sultan Karim, Dr. Ismail Laher, Dr. Ernest Puil, Dr. Craig Ries, Dr. Bhagavatula Sastry, Dr. Stephan Schwarz, Dr. Michael Walker and Dr. Richard Wall for all their advice and suggestions.

I would especially like to thank the members of my committee, Dr. David Godin and Dr. Catherine Pang, for all their advice and for taking the time to proofread my thesis. Last but not least, I would like to thank my two supervisors, Dr. Bernard MacLeod and Dr. David Quastel. I thank you for giving me this project and the opportunity to work with both you. Our many discussions were informative, heated and often long, but the lessons I learned I will cherish and never forget. I found working with both you to be a very rewarding experience. Thank you for a very memorable two years.

1. INTRODUCTION

1.1. Pulmonary Irritation

1.1.1. Definitions

The word irritation can be defined as an “extreme incipient inflammatory reaction of the tissues to an injury”, or the “evocation of a normal or exaggerated reaction in tissues by the application of a stimulus” (Spraycar, 1995). Pulmonary relates to the lungs (Spraycar, 1995). This must not be confused with the word respiratory, which relates to the act of respiration, defined as “a fundamental process of life, in which oxygen is used to oxidise organic fuel molecules, providing a source of energy as well as carbon dioxide and water” (Spraycar, 1995). So the term *pulmonary irritation* can be characterised as an exaggerated inflammatory reaction that is caused by the application of a stimulus in the lungs, in this case a pulmonary irritant; an irritant is a “substance which when inhaled, either causes tissue damage or stimulates nervous afferent end organs in the respiratory tract and lungs” (Widdicombe, 1977). Responses to the application of pulmonary irritants include apnea and coughing (Wang *et al*, 1996), bronchoconstriction (Widdicombe, 1977), and increased mucus secretion (Pon *et al*, 1994). The type of response is dependent on the concentration of the stimulant as well as the site of stimulation within the lungs (Widdicombe, 1977).

1.2. Anatomy and Physiology of the Pulmonary System

The inhalation of chemical irritants elicits reflexes which manifest themselves as symptoms described above. This is due to the stimulation of several receptor-types located in the respiratory tract lumen. Two of the more important receptors are the *rapidly adapting* and *C fibre receptors*.

1.2.1. *Rapidly Adapting Receptors*

The *rapidly adapting receptors* are also known as the *irritant receptors*, because they can be stimulated by exogenous and endogenous substances, such as histamine (Ganong, 1995; Widdicombe and Sant' Ambrogio, 1996). They are innervated by myelinated vagal fibres (Ganong, 1995) and were characterised as rapidly adapting because following continuous stimulation, their discharge was observed to be transient (Ganong, 1995). Chemical stimulation of these receptors in the nose, larynx, trachea and along the respiratory tract results in bronchoconstriction (to prevent deeper penetration of the irritant into the lungs) and also produces a cough or sneeze; the former occurs following stimulation in the trachea and the latter in the nose (Widdicombe and Sant' Ambrogio, 1996; Levitzky, 1999). In addition, stimulation of these receptors causes mucus secretion and hyperpnea (Ganong, 1995).

1.2.2. *C Fibre Receptors*

The *C fibre receptors* are believed to be free nerve endings innervated by unmyelinated vagal fibres (Ganong, 1995; Widdicombe and Sant' Ambrogio, 1996). They are classified into two groups, bronchial and pulmonary, depending on their location in the respiratory tract (Ganong, 1995). They are also known as *juxta-capillary receptors* because they are generally located near capillaries of the pulmonary circulation (Ganong, 1995; Widdicombe and Sant' Ambrogio, 1996). Stimulation of these receptors by endogenous or exogenous substances, such as capsaicin, induces apnea followed by rapid breathing, bradycardia, hypotension, bronchoconstriction and mucus secretion (Ganong, 1995; Widdicombe and Sant' Ambrogio, 1996). In addition,

stimulation of the bronchial C fibre receptors has been reported to induce coughing (Karlsson *et al*, 1988).

1.2.3. Other Receptor Subtypes

Another group of receptors found along the respiratory tract are the *slowly-adapting receptors*, which respond to lung inflation to shorten the inspiratory time and dilate the bronchi. They are also responsible for the Hering-Breuer inflation and deflation reflexes (Ganong, 1995; Widdicombe and Sant' Ambrogio, 1996; Levitzky, 1999). In addition the trachea and bronchi are innervated by cholinergic and adrenergic neurones (Ganong, 1995), as well as a number of noncholinergic and nonadrenergic neurones. The functional role of all these other receptor subtypes in reflexes due to inhaled irritants is not well understood (Ganong, 1995).

1.3. Methods of Evaluating Pulmonary Irritation

1.3.1. Animal Body Plethysmograph

It has been shown that stimulation of the upper respiratory tract with sensory irritants induces a reflex that results in a pause in exhalation, which decreases the breathing rate (Alarie, 1973; Vijayaraghavan *et al*, 1993). This reflex is mediated by stimulation of free nerve endings of the trigeminal nerve in the nasal mucosa (Ulrich *et al*, 1972; Barrow *et al*, 1977; Barrow *et al*, 1978; Alarie, 1981). This feature has been used as a quantitative measure to evaluate pulmonary irritants (Barrow *et al*, 1978; Vijayaraghavan *et al*, 1993; Hoymann and Heinrich, 1998).

The change in respiratory rate is measured with a plethysmograph. The animal is restrained in the plethysmograph with its head protruding into the exposure chamber

(head-only and nose/mouth-only exposure systems – see section 1.4.3, page 7) (Weyel *et al*, 1982). An inflatable collar with a comfortable airtight seal around the neck separates the exposure chamber from the rest of the animal's body (Barrow *et al*, 1977; Phalen *et al*, 1984). A rubber stopper seals the end of the plethysmograph, preventing the animal from escaping and creating a closed system where changes in pressure are measured using a pressure transducer (Barrow *et al*, 1977; Barrow *et al*, 1978).

With every inhalation, the pressure in the plethysmograph increases and vice versa for exhalation, which is then displayed on an oscillograph (Barrow *et al*, 1977). From the trace, the characteristic pause during exhalation (following stimulation of the free trigeminal nerve endings) is observed and used as an indicator of the net decrease in respiratory rate (Alarie, 1981). This method allows for continuous monitoring of the animal prior to, during and following exposure to an irritant (Barrow *et al*, 1977; Alarie, 1981). Altering the concentration of the irritant leads to a concentration-response relationship, where the RD50 (the concentration of irritant needed to decrease the respiratory rate by 50%) is determined and used as an index of discomfort (Barrow *et al*, 1978; Alarie, 1981).

1.3.2. *Measuring Cough*

Another commonly used method of evaluating pulmonary irritation is by measuring the number of induced coughs within a certain time period (Zelenak *et al*, 1982). This method is relatively simpler compared to the previous one. Animals (usually guinea pigs) are placed in a whole-body exposure chamber (section 1.4.3.4, page 9) with the irritant inside (Zelenak *et al*, 1982; Featherstone *et al*, 1996). The number of elicited coughs in a particular time period are recorded either by direct observation or a

microphone (Zelenak *et al*, 1982; Featherstone *et al*, 1996; Doherty *et al*, 1997). By analysing the acoustic properties of the coughs, it is possible to distinguish between coughs in normal individuals and irritant-induced coughs (Doherty *et al*, 1997).

1.3.3. Biochemical Methods of Evaluating Irritants

It has been reported that following stimulation of the respiratory tract with irritants such as capsaicin or hypertonic saline, increased vascular permeability, plasma extravasation and tissue swelling were observed; this was termed *neurogenic inflammation* (Joos *et al*, 1995). It has also been reported that inflammatory mediators are released from mast cells (Blom *et al*, 1997) following stimulation. Therefore, an additional method of evaluating the effect of irritants on the pulmonary system is to collect nasal lavage samples (Philip *et al*, 1996; Blom *et al*, 1997). These samples undergo cytologic analysis to determine the number of leukocytes, or a differential cell count (Philip *et al*, 1996). These samples also undergo a radioimmunoassay to determine the levels of leukotrienes and prostaglandins (Blom *et al*, 1997).

A more invasive method is to remove the lungs of the animals following exposure to the irritant and homogenise them (Pon *et al*, 1994). The particulate matter is then centrifuged, chromatographed and stained for particular mucosubstances (Pon *et al*, 1994). All these methods provide evidence for the biochemical effects of irritants on lung tissues.

1.4. Exposure Systems

In order to evaluate pulmonary irritants, animal subjects have to be exposed to them under controlled conditions. There are a variety of exposure systems that are

currently used. They are classified into three general categories: static, recirculating and dynamic exposure systems (Cheng *et al*, 1995).

1.4.1. *Static Exposure Systems*

In this system, test atmospheres are produced by placing a finite amount of test substance into a closed chamber (MacFarland, 1983; MacFarland, 1987). There are several advantages to this system over the other ones, namely (1) that consumption of test substance is minimised, and (2) since there is no flow through the chamber, consistent generation of aerosols or gases of the test substance is not required (Cheng *et al*, 1995). However, there are also several disadvantages: (1) oxygen concentration depletes in the chamber, (2) the temperature rises, and (3) the airborne concentration of the test substance decreases as a result of deposition on the surface of the exposure chamber and experimental animals (Phalen *et al*, 1984; Cheng *et al*, 1995).

1.4.2. *Recirculating Exposure Systems*

This system is similar to the static exposure system; however, it employs a loop that removes water and carbon dioxide from the chamber and adds fresh oxygen. The disadvantage, similar to the static chamber, is that a steady concentration of the test substance cannot be maintained. It has also been reported that the loss of test substance has been higher in this system compared to the static exposure system (Cheng *et al*, 1995).

1.4.3. Dynamic Exposure Systems

In this system, the test substance is continuously replenished, i.e. it is delivered to and exhausted from the exposure chamber at a constant flow; the test substance is not recirculated in a loop (MacFarland, 1987; Cheng *et al*, 1995). As a result, following an initial increase in the amount of test substance in the chamber, a steady concentration is sustained (MacFarland, 1983).

The main advantages of this system are that the temperature and relative humidity are maintained at a constant level, oxygen is continuously replenished and carbon dioxide is removed (Cheng *et al*, 1995). However, this system does require large quantities of the test substance compared to the other two exposure systems. It also requires other items such as pumps, compressed air and vacuum sources (Cheng *et al*, 1995). In general, the dynamic exposure chamber is the preferred system by most investigators that study various aspects of animals' pulmonary system (MacFarland, 1983; Phalen *et al*, 1984).

There are several categories of dynamic exposure systems that are commonly used:

1.4.3.1. Head-Only Exposure Chamber

The main purpose of this system is to expose the animals to the irritant while minimising contact of the substance with the skin; only the animal's head is exposed to the test substance. (MacFarland, 1983). The animal is placed in a cylinder which would be separated by either an inflatable collar or a thin rubber membrane that stretches to accommodate the neck (Phalen *et al*, 1984). The test substance would then be introduced into side of the cylinder with the animal's head and the substance would not

be exposed to the rest of the body. Some advantages of this system include the small size of the exposure chamber and small amount of test substance that is required (Cheng *et al*, 1995). However, the small size of the exposure chamber does have several limitations, such as difficulty in maintaining proper air temperature, humidity, carbon dioxide levels and surrounding noise (Phalen *et al*, 1984). As well, because this system utilises a collar with a seal around the neck, the animal is firmly restrained, and the resulting stress may be significant (Phalen *et al*, 1984). There is also the possibility of choking the animal if the collar were too tight.

1.4.3.2.Mouth/Nose-Only Exposure Chamber

This system is very similar to the head-only exposure chamber, except that it employs a mask that is placed around the mouth and nose of the animal. Here the exposure of the test substance is limited to the nose and mouth of the animal, thus eliminating any possible eye irritation (Phalen *et al*, 1984). Additional advantages include a smaller requirement and easier containment of the test substance within the exposure apparatus, and the possibility of rapidly altering the concentration of the test substance (Phalen *et al*, 1984). There are, however, several disadvantages, which include obtaining a tight seal around the mask and considerable stress on the animal due to the close confinement (Phalen *et al*, 1984).

1.4.3.3.Partial Lung/Lung-Only Exposure Chamber

In this system, the test substance is administered directly to the lungs via an intratracheal catheter. The advantages of this system are that there is only one route of exposure, less of the substance is needed and it eliminates the exposure of other parts

of the body (eyes and skin). However, disadvantages of this system include technical difficulties because the animals must be anaesthetised and catheterised, the nose is bypassed and the animals experience a great deal of stress (Phalen *et al*, 1984).

1.4.3.4. Whole-Body Exposure Chamber

Here the whole animal is exposed to the test substance. Advantages of this system include minimal animal restraint, it is a well-controlled environment, it can generate stable concentrations of the test substance and it is capable of exposing the substance to a number of animals and different species at the same time, depending on the size of the exposure chamber (Phalen *et al*, 1984; MacFarland, 1987; Cheng *et al*, 1995). Some of the disadvantages include a large requirement of the test substance, its deposition on the surface of the chamber and the bodies of the animals, possible interactions of the substance with animal excreta, and difficulty in obtaining a uniform distribution of the substance in the chamber (Phalen *et al*, 1984, Dorato and Wolff, 1991). In order to reduce adsorption of the test substance and corrosion of the exposure chamber, they are commonly constructed out of glass or stainless steel (MacFarland, 1987; Dorato and Wolff, 1991).

At a workshop organised under the auspices of the Ministry of Health, National Institute of Health Research and Development in Jakarta, Indonesia, where the evaluation and assessment of toxicity of inhaled household insecticides was discussed, the majority of participants indicated that the head/nose-only exposure system was the preferred choice (Achmadi and Pauluhn, 1998). However, it has also been considered that because the whole human body is exposed to household insecticides, and not only the head and/or nose, the whole-body exposure system should be the first choice

(Achmadi and Pauluhn, 1998) because it would have more direct extrapolations to human responses.

1.5. Factors to Consider with Exposure Systems

There are a number of factors that have to be taken into account when choosing an appropriate exposure system. These include temperature, humidity, whether a gas or aerosol will be used, etc...

1.5.1. Temperature

Experimental animals generate heat as a result of their metabolic processes. With many animals in the exposure chamber at the same time, the increase in heat can be substantial (MacFarland, 1983). It has been observed that responses from animals differ depending on the ambient temperature (Salem, 1987); a change in temperature might influence the severity, duration, nature and variance of a response to a test chemical (Cheng *et al*, 1995). Thus, a constant temperature must be maintained in the exposure chamber. This is achieved by limiting the number of animals that are being placed in the chamber. It has conventionally been decided that the total volume of all the animals in the chamber not exceed 5% of the chamber volume (Salem, 1987; Cheng *et al*, 1995).

1.5.2. Relative Humidity

The relative humidity in the exposure chamber is also important because it can influence behaviour and chemical effects on animals (Cheng *et al*, 1995), as well as the particle size of hygroscopic aerosols and concentrations in the chamber (Salem, 1987).

In addition, it is also important in maintaining heat balance. Thus, chambers (dynamic exposure system) have to be ventilated with air of about 35 – 40% relative humidity (Cheng *et al*, 1995).

1.5.3. *Uniform Distribution*

The test substance must be uniformly distributed within the exposure chamber in order to have all the experimental animals exposed to the same amount of test substance and to reduce an experimental source of variance.

1.5.4. *Deposition of the Test Substance on the Animal's Fur*

This is another reason for limiting the number of animals that are in the chamber at any one time. With large numbers of animals in the chamber, there is an increased surface area for the test substance to deposit itself. As a result, concentrations of the test substance in the atmosphere decrease. Thus, again the suggested volume of animals in the chamber should not exceed 5% of the chamber volume (Silver, 1946; Cheng *et al*, 1995).

1.5.5. *Types of Test Substances*

There are a number of methods that can be utilised to administer the test substance. The easiest of these is to have it as a gas. However, not all substances can be administered in gaseous form. Thus, a common method is to nebulize the test agent and administer it as an aerosol.

1.5.5.1. Ultrasonic Nebulizers

There are several methods of generating aerosols. A common method is to use an ultrasonic nebulizer. The aerosol is generated by high frequency oscillations (typically 1 – 3 MHz) from a piezoelectric crystal located beneath the reservoir containing the test substance in liquid form (Newman, 1993). This produces a spray of droplets which are then propelled by an air stream into the exposure chamber (Marple and Rubow, 1979). The advantage of this system is that it produces aerosol particles with Mass Median Aerodynamic Diameters (MMAD) of around 5 μm , which is necessary if the aerosol is to be inhaled by animals and be able to reach the alveoli (Newman, 1993, Conley *et al*, 1997); aerosols with smaller MMADs (1 – 2 μm) are preferable (Lewis *et al*, 1989). Large droplets are returned to the reservoir to be renebulized. Unfortunately, these systems are large and bulky, because they need ultrasonic generators, and they require electric power (Newman, 1993).

1.5.5.2. Jet Nebulizers

Another common method is to utilise a jet nebulizer, which is based on the Bernoulli principle. A compressed stream of air passes through a narrow constriction (a venturi), increasing its velocity, causing a decrease in pressure. This change in pressure is sufficient to draw liquid from a reservoir and propel it along with the stream of compressed air into the exposure chamber (Marple and Rubow, 1979; Newman, 1993). Aerosols generated by jet nebulizers have MMADs within the range of 1 to 5 μm (Marple and Rubow, 1979). A number of studies have been performed in which commercially available jet nebulizers were compared in their aerosol generation (Hess *et al*, 1996; Conley *et al*, 1997). It was found that they all produced aerosol particles

that were similar in diameter, with a range from 1.94 to 3.84 μm (Conley *et al*, 1997) and that the optimal delivery of aerosol depends on the volume of fluid in the reservoir, the flow rate and brand of nebulizer (Hess *et al*, 1996).

The advantages of using jet nebulizers are that they are relatively smaller compared to ultrasonic nebulizers and they do not require either heavy ultrasonic generators or electricity. They can also nebulize large concentrations of almost any substance (as long as it is in liquid form). However, following prolonged use, a significant proportion of the test substance remains on the internal walls of the nebulizer. This *residual volume* becomes progressively more concentrated (Newman, 1993). Thus, the concentration of the test substance in the aerosol will diminish.

1.5.5.3. Other Methods

There are a number of other methods of generating aerosols, such as the Metered-Dose Inhalers. These are most commonly used to administer bronchodilator agents directly into the lungs of asthmatic patients (Newman, 1993). Other less known methods include the dry powder inhaler, spinning-disc atomizer, vibrating-orifice aerosol generator and a condensation generator (Marple and Rubow, 1979; Newman, 1993).

1.5.5.4. Aerosol Dosimetry

There is one disadvantage to using any type of aerosol generator. The concentration of the test substance in the exposure chamber can be readily determined. However, that is not the case with the concentration of the substance in the lungs. The delivered dose depends on a number of factors, including exposure concentration, intake rate, deposition and retention (Dorato and Wolff, 1991). Thus, the

actual amount of test substance that the lungs are exposed to is never known. This unfortunately cannot be rectified without conducting very invasive procedures. Common methods of dealing with this difficulty are to reduce other sources of variance by maintaining a constant volume of the test substance in the nebulizer, maintaining a constant flow rate, and controlling all the other above-mentioned factors. Conventionally, the literature correlates responses to either the concentration of the test substance in the exposure chamber or the concentration in the nebulizer.

1.6. Rationale for the Study

Even though all the above methods of evaluating pulmonary irritants have been used for many years, and they are valid and approved by animal ethics boards, these methods have a general disadvantage of putting a great deal of stress on the experimental animals. Stress can be defined as a physical or psychological stimulus that disturbs the normal physiological equilibrium (Spraycar, 1995). In the animal plethysmograph assay (section 1.3.1, page 3), the animal is restrained; it has no chance of escaping from the noxious atmosphere that it is forced to breathe. For the method in which the number of coughs are measured (section 1.3.2, page 4), the animal is not restrained (it is free to move about in the exposure chamber); however, it cannot escape the test substance. The other methods (section 1.3.3, page 5) either subject the animals to fairly invasive procedures, or require the investigator to sacrifice the animals in order to remove their lungs. All these procedures put a significant amount of stress on the animals.

We propose a new assay for evaluating pulmonary irritation based on the principle of avoidance. The objective was to develop a quick and easy assay to

evaluate pulmonary irritation without subjecting the animals to a relatively high level of stress.

2. MATERIALS & METHODS

2.1. Apparatus

The Minimal Animal Stress Irritant Assay Chamber (MASIAC) consists of a plexiglas cylinder, divided by a disc, with an open central door through which a mouse can pass freely (figure 1). With a 4.5-inch diameter and a 24-inch length, it has a volume of roughly 5.5 L. The aerosols are introduced into the chambers through distal inlets by way of nebulizers (VixOne Nebulizer, Westmed Inc.); they flow perpendicular to the long axis in a helical fashion towards the central disc, where the outlets are located. This allows for rapid and complete mixing in each chamber. An irritant, dissolved in distilled water, is placed in one nebulizer and water in the other; both are nebulized with compressed air at a flow rate of 5 L/min. If the mice feel any discomfort or irritation in the Test (irritant) Chamber, they are free to move into the Non-Test (non-irritant) Chamber. All experiments were performed in a fume hood so as to minimise the exposure of the experimenter to the irritants.

2.2. Animals and Drugs

Young female CD-1 mice (25 – 30 g) were used for the experiments because they are small and inexpensive to purchase and maintain, as compared to other laboratory animals; females were chosen because Dicpinigaitis and Rauf reported that healthy women were more sensitive to inhaled irritants than men (Dicpinigaitis and Rauf, 1998). The mice were obtained from Charles River, Quebec, Canada. All experimental procedures on the mice were approved by the University of British Columbia Animal Care Committee. Citric acid (Sigma Chemical, Poole, UK) and sodium

Figure 1: (a) Schematic Diagram of the Minimal Animal Stress Irritant Assay Chamber (MASIAC); (b) Picture of the MASIAC with Mice.

metabisulfite (Nichols Chemical Company, Montreal, Canada) were dissolved in distilled water and diluted to the concentrations used for the experimental procedures.

Ammonia (household ammonia purchased from a local hardware store) was diluted in distilled water to the required concentrations. Capsaicin (Research Biochemical International, Natick, MA, USA) was dissolved in ethanol and diluted with distilled water. Morphine (Abbott, Toronto, Canada) was diluted with normal physiological saline and injected into the mice (ip). Capsazepine (Research Biochemical International, Natick, MA, USA) was dissolved and diluted in 20% intralipid emulsion (Research Biochemical International, Natick, MA, USA) and injected (ip) into the mice.

2.3. Method I

Six randomly chosen mice were placed in the MASIAC and at time 0 minutes, the compressed air was turned on. A single concentration of irritant was used. At time 5 minutes, the number of mice in each chamber was recorded. At this point, the aerosols were reversed; the aerosol that was introduced into the right chamber was now blown into the left chamber and vice versa. The atmospheres equilibrated with the incoming aerosols and at time 10 minutes, the number of mice in each chamber was recorded again. This was done in order to test whether the mice had a preference for one of the chambers and whether they moved between the chambers as a result of the irritant aerosol. The two measurements were averaged and presented as the mean $\pm$ SEM, and analysed using a Student's t Test.

2.4. Method II

With eight randomly-chosen mice in the MASIAC, the duration of the experiment was shortened to 5 minutes and the number of mice in both chambers was recorded at 3.0, 3.5, 4.0, 4.5, and 5.0 minutes after the compressed air was turned on. The average of the five counts provided a number indicating which chamber the mice preferred to be in.

Starting with a low irritant concentration (after a control), a 5-minute experimental run was performed. Following the run, the MASIAC chamber was cleaned and with the same group of eight mice, another 5-minute run was carried out with a quadrupled irritant concentration, and then another, until a concentration was reached which resulted in all the mice avoiding the Test Chamber.

2.4.1. Justification for Time Points

In dynamic exposure chambers, the time it takes for the internal atmosphere to equilibrate is dependent on the volume and total flow rate through the chamber (MacFarland, 1983; Phalen *et al*, 1984; Lewis *et al* 1989 and Cheng *et al*, 1995). The formula that describes this relationship is:

$$t_x = k \frac{V}{F}$$

where:

t_x	=	the time required to attain x% of the equilibrium concentration
k	=	a constant whose value is determined by the value of x
V	=	the total volume of the chamber
F	=	total flow rate through the chamber

Conventionally, t_{99} is calculated, i.e. the time that is required to reach 99% of the equilibrium concentration. The constant, k , for t_{99} is 4.6 (Cheng *et al*, 1995). In the case of the MASIAC, the total flow rate was 10 L/min (5 L/min going into each chamber) and the total volume was 5.5 L. Therefore, the time required to reach 99% of the equilibrium concentration was:

$$\begin{aligned} t_{99} &= 4.6 \frac{5.5}{10.0} \\ &= 2.53 \text{ minutes} \end{aligned}$$

Thus, it took about two and a half minutes for the atmospheres in the MASIAC to equilibrate with the aerosols, and thus the first measurement was taken at 3.0 minutes. In fact, from observing the aerosols flowing into the chambers, the time it took for the atmospheres to equilibrate appeared to be shorter. This was due to the cylindrical shape of the MASIAC which facilitated a more efficient flow of the aerosol through the two chambers. However, because extensive mathematical calculations would have been required to calculate the time for equilibration, it was decided that the best course of action would be to use the standard formula for dynamic exposure chambers.

2.4.2. Calculation of Percent Irritation

The percent of mice that were irritated by the aerosol represents the net percent of mice that avoided the Test Chamber, i.e. the percent of mice that were in the Non-Test Chamber minus 50% multiplied by 2.

$$\% \text{ Irritation} = 2 (P_{NT} - 50)$$

where P_{NT} = percent of mice in the Non-Test Chamber

The reason for this was that when the mice did not feel any discomfort, they were equally distributed (4 in the Non-Test and 4 in the Test chambers). That was defined as 0% irritation. When there were 6 mice in the Non-Test and 2 in the Test Chambers (75% of mice in the Non-Test Chamber), that was expressed as 50% irritation, and so on.

2.4.3. Calculation of EC50

The mean percent irritation ($\pm$ SEM) from the 5 measurements was plotted against the concentration of the irritant in the nebulizer, resulting in a dose-response relationship. Using the GraphPad Statistical Analysis Program, a curve was fitted to the data points and the EC50 value ($\pm$ SEM) was determined from that curve.

2.5. Modifying the MASIAC Chamber

One of the problems with the above Method II was that to show irritation, the number of mice avoiding the Test Chamber had to increase from four to eight. Because that is a relatively small difference, there was concern that many animals would have to be tested in order to show the difference to be significant. Therefore, in an attempt to eliminate that concern, the MASIAC chamber was modified.

In the original MASIAC, the central disc was in the middle, making both the Test and Non-Test Chambers symmetrical; during baseline conditions, the mice were equally distributed between the two chambers. The central disc was repositioned three quarters of the way between the outer doors, making one chamber three times larger than the other; the larger was designated the Test Chamber and the smaller the Non-Test Chamber. As a result, there were more mice in the Test Chamber as compared to

the Non-Test Chamber during baseline conditions, resulting in a larger requirement of mice avoiding the Test Chamber to show irritation.

However this design was deemed unreliable since by observing the flow of the aerosols in the chambers, it was noted that in the Test (large) Chamber, the aerosol did not mix uniformly; it flowed in a helical fashion from the inlet to the centre of the MASIAC (the position where the disc was located in the symmetric design). From that point on, the aerosol seemed to drift towards the disc, suggesting that the aerosol concentration in that area of the chamber was significantly reduced. In addition, it was noticed that the mice tended to huddle together in that area where the diluted aerosol was present. This was not observed in the symmetrical MASIAC design. It was thus concluded that the original symmetrical design be used for all experiments.

2.6. Method III

The number of mice tested simultaneously was increased to ten per group. At each of the 5 measurements during every 5-minute run, in addition to recording the number of mice in either chamber, the identity of each mouse was also noted. Then, at each measurement time, each individual mouse was given a score of 0 if it was in the Test Chamber, or 1 if it was in the Non-Test Chamber. Adding up the scores, each mouse had a maximum Test Chamber Avoidance Score (TCAS) of 5 for each 5-minute run at each concentration of irritant aerosol; a score of 5 representing maximum irritation. This method gave a number indicating which chamber each mouse, rather than the whole group, preferred to be in.

2.6.1. Calculation of EC50

For graphing purposes, plotting the averaged score of all the mice versus the concentration of the irritant gave a dose-response relationship (the dose is defined as the concentration of the irritant in the nebulizer). A curve was fitted to the data points (using a score of 5 as the maximum and the expected baseline score of 2.5 as the minimum) and an EC50 value ($\pm$ SEM) was determined the same way as in Method II above (section 2.4.3, page 21). However, since each mouse had a score for each concentration of the irritant, for analytical purposes, a dose-response curve was plotted for each individual mouse. Using the GraphPad Statistical Analysis Program, an EC50 value was calculated from those curves for each individual mouse, which were then averaged resulting in a mean EC50 ($\pm$ SEM) for irritation.

There was a small discrepancy in the results that were determined between these two methods; the EC50s were similar to each other, but the standard errors from the graphical method were larger than the analytical one. Therefore, it was decided to report the EC50s that were obtained using the latter method.

3. RESULTS

3.1. Method I

3.1.1. Response to 1.4 M Citric Acid

Citric acid is a standard pulmonary irritant and is commonly used to evaluate pulmonary irritation (Lalloo *et al*, 1995 and Featherstone *et al*, 1996). A citric acid concentration of 1.4 M in the nebulizer was chosen because it has been published that exposing mice to this aerosol caused reproducible irritation (Featherstone *et al*, 1996).

Figure 2 shows the averaged responses of one group of six mice to the 10-minute experimental run of water and 1.4 M citric acid aerosol in the nebulizer. The results are expressed as the mean of the two measurements $\pm$ SEM; the average numbers of mice avoiding the Test Chamber with water and citric acid aerosol were 3.5 ± 0.5 and 5.5 ± 0.5 , respectively. The figure indicates that with water aerosol, the mice were roughly equally distributed between the chambers, and the 1.4 M citric acid aerosol caused discomfort in the animals as evidenced by the fact that the majority of the mice avoided it.

3.1.2. Absence of Desensitisation

The literature indicated that when human beings were continuously exposed to citric acid, desensitisation occurred (Morice *et al*, 1992). To determine if desensitisation occurred with citric acid in mice, and to determine whether the results were reproducible, the following experiment was performed. With six mice per group, the 10-minute experimental run with 1.4 M citric acid was performed only once on six separate groups of mice. Then the same 10-minute experiment was carried out six times in

Figure 2: Exposure of Six Mice to an Aerosol of Distilled Water and a Single Concentration of Citric Acid. Each bar is the mean ($\pm$ SEM) of the number of mice avoiding the Test Chamber.

Multiple Testing with 1.4 M Citric Acid

Figure 3: Multiple Testing with 1.4 M Citric Acid. In the first column, six different groups of mice (6 mice per group) were tested once. In the second column, one group of mice was exposed to 1.4 M citric acid six times. Both bars represent the average number of mice that avoided the Test Chamber ($\pm$ SEM).

succession on a single, naive group of mice. The data were tested for normality and then analysed using a Student's t test.

Figure 3 indicates that the responses of the six different groups of mice were similar (the average number of mice avoiding the Test Chamber, expressed as the mean $\pm$ SEM, was 5.3 ± 0.2), suggesting that the results were reproducible. In addition, comparing those results with the one group of mice that was exposed six times (average number of mice avoiding the Test Chamber was 5.5 ± 0.3), the responses were similar, indicating that the mice did not experience desensitisation ($p > 0.5$).

However, 1.4 M citric acid was a relatively high concentration; it was not known where on the citric acid dose-response curve 1.4 M was located. Therefore, it was decided that a dose-response experiment to citric acid be carried out instead of testing a single concentration.

3.2. Method II

One noted observation was that the mice continuously moved between the chambers. Thus, the two measurements at 5 and 10 minutes (Method I) did not represent the movement of the mice during the course of the experiment. In addition, after 5 minutes the chambers were dirty with aerosol deposits. Reversing the chambers and having, for example, water aerosol flowing into a chamber previously filled with the irritant might have affected the responses of the mice, even after the 5-minute equilibration period. Therefore, the method was changed as described in section 2.4 (page 19). Additionally, it was found that there was no significant difference in the results between reversing the chambers and not reversing them for each subsequent irritant concentration, therefore, they were not reversed as in Method I.

3.2.1. *MASIAC Control Experiments*

Using Method II, the following control experiments were performed. In the first experiment, there was no air flowing through the MASIAC itself (this was to determine if the mice preferred either of the two chambers when nothing was flowing through them). In the second and third experiments, air or water aerosol flowed through both chambers, respectively. In the fourth control experiment, air flowed into the Non-Test Chamber and water aerosol into the Test Chamber. Each experiment was performed three times on different groups of eight mice. Following the determination that the data were normally distributed with equal variances, the results were analysed by an analysis of variance.

Figure 4 shows that with nothing flowing through the chambers, the average number of mice avoiding the Test Chamber was 3.9 ± 0.3 . With air or water flowing through the chambers, the numbers of mice avoiding the Test Chamber were 4.0 ± 0.2 and 3.9 ± 0.2 , respectively. In the fourth control experiment, in which water aerosol was introduced into the Test Chamber and air into the Non-Test Chamber, the number of mice avoiding the Test Chamber was 4.2 ± 0.3 . The figure indicates the mice were about evenly distributed between the chambers ($p > 0.5$). The results are expressed as the average ($\pm$ SEM) of the number of mice avoiding the Test Chamber (5 measurements from 3 groups).

3.2.2. *Citric Acid and Capsaicin Dose-Response Curves*

Following the control experiments, dose-response curves were performed on two groups of mice with two standard pulmonary irritants, citric acid and capsaicin. Figures

MASIAC Control Runs

Figure 4: MASIAC Control Runs. With eight mice in the MASIAC, the following control experiments were performed. In the first one, the compressed air was turned on, but the airflow was not directed through the chambers. In the second and third experiments, air or water aerosol were introduced into both chambers, respectively. In the fourth experiment, air was flowing into the Non-Test Chamber and water aerosol into the Test Chamber. Each experiment was performed three times. The results are expressed as the average number of mice avoiding the Test Chamber $\pm$ SEM (5 measurements over 3 groups).

5 and 6 show the dose-response curves to those two irritants. Each point on the graphs is the mean ($\pm$ SEM) percent irritation that the mice experienced over the five measurements. The right sides of the graphs have the corresponding number of mice that avoided the Test Chamber. The EC50 value for citric acid was calculated to be 21.6 ± 2.6 mM and for capsaicin 0.95 ± 0.09 mM.

It has been published that a citric acid concentration of 0.25 M elicited cough and nasal irritation in guinea pigs (Lalloo *et al*, 1995). The MASIAC detected pulmonary irritation due to citric acid at a much lower concentration, suggesting that it could be more sensitive than currently used assays. For capsaicin, many different concentrations have been used to induce cough and irritation. They ranged from 0.03 to 4.9 mM (Palecek *et al*, 1989; Pinto *et al*, 1995; Bolser *et al*, 1995; Lalloo *et al*, 1995; Bootle *et al*, 1996). The results obtained with the MASIAC indicate that they were within that range.

3.3. Method III

Method III gave a number indicating which chamber each mouse preferred to be in. In addition, since the analytical procedure for calculating the EC50 gave a mean with a smaller error (compared to the graphical one), Method III was used for all remaining experiments (section 2.6, page 22). In addition, the symmetric MASIAC chamber was used for the reasons outlined in section 2.5 (page 21).

3.3.1. Dose-Response Curves to Four Pulmonary Irritants

Using Method III, dose-response curves were performed for citric acid, capsaicin,

Citric Acid Dose-Response Curve

Figure 5: Citric Acid Dose-Response Curve. Each point is the mean ($\pm$ SEM) percent irritation that the mice experienced over the five measurements. The right side of the graph has the corresponding number of mice that avoided the Test Chamber. Dose is defined as the concentration of irritant in the nebulizer.

Capsaicin Dose-Response Curve

Figure 6: Capsaicin Dose-Response Curve. Each point is the mean ($\pm$ SEM) percent irritation that the mice experienced over the five measurements. The right side of the graph has the corresponding number of mice that avoided the Test Chamber. Dose is defined as the concentration of irritant in the nebulizer.

sodium metabisulfite and ammonia on groups of 10 mice. Plotting the averaged TCAS score of the mice versus the concentration of the irritant in the nebulizer gave a dose-response curve, with a baseline score of 2.5 in the absence of irritant. Figures 7, 8, 9, and 10 show the respective dose-response curves, with the dotted line representing the baseline TCAS score of 2.5. The EC₅₀ value for citric acid was 27.4 ± 5.3 mM, for capsaicin 0.42 ± 0.15 mM, for sodium metabisulfite 19.2 ± 3.5 mM, and for ammonia 70.8 ± 13.2 mM.

3.3.1.1. Distribution of Citric Acid EC₅₀ Values

Citric acid EC₅₀s from experiments performed on 112 mice were plotted on a cumulative histogram in order to examine their distribution; panel (a) of figure 11 indicates that the EC₅₀ values were not normally distributed. Testing the data with the Kolmogorov-Smirnov Test resulted in a statistically significant deviation from normality ($p < 0.005$). However, plotting the log₁₀-transformed EC₅₀s resulted in a normal Gaussian distribution (panel (b) of figure 11), with a non-significant Kolmogorov-Smirnov statistic ($p > 0.1$). The mean of the 112 log₁₀ citric acid EC₅₀s was -1.57, with a standard deviation of 0.48. Dividing the deviation by the square root of the number of mice per group, and taking the anti-log₁₀, predicts a standard error range for the mean EC₅₀; with a group of 10 mice, the error would be expected to be in a range within about 40% of the mean EC₅₀ value.

3.3.2. Group Effect

With a group of mice in the chamber at the same time, it would seem possible that the mice would tend to follow one another; this would either invalidate the assay or

Citric Acid Dose-Response Curve

Figure 7: Citric Acid Dose-Response Curve. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 value is the mean ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer.

Capsaicin Dose-Response Curve

Figure 9: Capsaicin Dose-Response Curve. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 value is the mean ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer.

Sodium Metabisulfite Dose-Response Curve

Figure 9: Sodium Metabisulfite Dose-Response Curve. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 value is the mean ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer.

Ammonia Dose-Response Curve

Figure 10: Ammonia Dose-Response Curve. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 value is the mean ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer.

(a)

(b)

Figure 11: Cumulative Distribution Histograms of Citric Acid EC50 Values. In panel (a), EC50s from 112 mice were plotted on a cumulative histogram. In panel (b), the $\log_{10}$ -transformed EC50s from the same 112 mice were plotted.

necessitate a relatively complicated statistical analysis. To test for this "positive" group effect, a correlation analysis was performed on each mouse's movement compared to the movement of the rest of the mice in the chamber. Results from a 5-minute run at a low irritant concentration were used because it had a negligible effect on the avoidance reaction (TCAS score of around 2.5), while at higher concentrations, all the mice avoided the Test Chamber.

From every 5-minute run, each mouse had five scores of either 0 or 1 (section 2.6, page 22). A correlation was performed between each mouse's score and the sum of the scores of the rest of the mice at each of the 5 measurements; the correlation *r*-value was calculated for each individual mouse. This analysis was performed on runs in which low concentrations of citric acid, capsaicin, sodium metabisulfite, ammonia or water aerosol were administered into the Test Chamber, as well, with randomly generated mouse avoidance scores.

Table 1 gives an example of results obtained from one experimental run with a citric acid concentration of 2.4 mM. Also displayed are the *r*-values from the correlation analysis that was performed on the movement of the mice. Averaging the *r*-values from 50 mice, it was found that in the presence of a low concentration aerosol of any of the four pulmonary irritants, there was a small "negative" group effect (a negative correlation was found), i.e. the mice had a tendency to avoid each other (table 2). However, when the results from experiments with water aerosol were analysed, it was found that there was no correlation, as with randomly generated mouse avoidance scores. These results suggest that an aerosol irritant, even at low concentrations, affects the mice such that they avoid clustering. At the higher concentrations, the noxious effect of the aerosol overcomes this desire to avoid other mice and they huddle

Table 1: Results from One 5-minute Experimental Run with a Citric Acid Concentration of 2.4 mM.

	Time (min)					Correlation R-Value
	3.0	3.5	4.0	4.5	5.0	
Mouse 1	1	0	0	0	1	0.76
Mouse 2	0	1	1	0	1	-0.27
Mouse 3	0	0	0	0	1	0.56
Mouse 4	0	0	0	1	1	-0.21
Mouse 5	1	0	0	1	1	0.08
Mouse 6	1	1	1	1	0	-0.87
Mouse 7	0	1	0	0	1	0.17
Mouse 8	1	1	0	1	0	-0.54
Mouse 9	1	1	1	0	0	-0.54
Mouse 10	1	0	1	0	1	0.08

Above are the avoidance scores of the mice at 3.0, 3.5, 4.0, 4.5, and 5.0 minutes after turning on the compressed air. For every experimental run, a correlation was performed between one mouse's avoidance score at each measurement and the sum of the scores of the rest of the mice at that same measurement. The correlation r-values were calculated for each individual mouse and are shown in the right column (bolded).

Table 2: Averaged Correlation R-Values from 50 Mice under Different Experimental Conditions.

Test Chamber Aerosol	Number of Mice	Average Correlation R-Value
Low [citric acid]	50	- 0.20 $\pm$ 0.07
Low [capsaicin]	50	-0.13 $\pm$ 0.07
Low [Na metabisulfite]	50	-0.23 $\pm$ 0.08
Low [ammonia]	50	-0.08 $\pm$ 0.07
Distilled water	50	0.01 $\pm$ 0.06
Randomly generated avoidance scores	50	0.01 $\pm$ 0.07

Correlation r-values from 50 mice that were tested at each of the experimental conditions (left column) were averaged together (displayed as the mean $\pm$ SEM). A small negative correlation ("negative" group effect) was found between the movement of the mice in the presence of low concentrations of the four irritants, but no correlation was found with water aerosol in the Test Chamber, as with randomly generated avoidance scores.

to avoid the irritant. Therefore, the mice had no significant tendency to cluster together, thus the testing of a group of mice simultaneously is valid.

3.3.3. *Modification of the Dose-Response Curves by Morphine and Capsazepine*

Morphine, an opioid analgesic, has been used to reduce the irritation due to pulmonary irritants at a dose of 10 mg/kg (Fuller *et al*, 1988; Dambisya *et al*, 1992). As well, capsazepine, a selective antagonist of the capsaicin receptor, has been used as an analgesic in animal pain models at doses up to 37.6 mg/kg (sc)(Bevan *et al*, 1992; Perkins and Campbell, 1992; Santos and Calixto, 1997). With that in mind, these agents were used to investigate whether or not the MASIAC could detect their adjustment of the irritant dose-response curves.

With ten mice being tested together, five of them were injected with one of the drugs and the other five with vehicle control (saline for morphine and 20% intralipid emulsion for capsazepine) 15 minutes prior to the start of the experiment. Each experiment was conducted on two groups of mice and the results were combined such that there were a total of ten mice in both the control and antagonists groups. The experimenter was blinded as to which mouse got what. The two groups were compared by analysing the $\log_{10}$ EC50s with a Student's t Test.

Figures 12 through to 19 show the shift in the irritant dose-response curves by 10 mg/kg morphine and 18.8 mg/kg capsazepine injected (ip). Table 3 summarises the results, presenting the mean EC50 values for the control mice as well as the antagonist mice. The p-values from a Students t Test comparing the $\log_{10}$ EC50s between the two groups are also displayed.

Citric Acid Dose-Response Curves

Figure 12: Citric Acid Dose-Response Curves with and without 10 mg/kg Morphine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 10 mg/kg morphine and the other five with saline. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was a statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p < 0.02$).

Citric Acid Dose-Response Curves

Figure 13: Citric Acid Dose-Response Curves with and without 18.8 mg/kg Capsazepine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 18.8 mg/kg capsazepine and the other five with intralipid vehicle. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was a statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p < 0.05$).

Figure 14: Capsaicin Dose-Response Curves with and without 10 mg/kg Morphine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 10 mg/kg morphine and the other five with saline. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was a statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p < 0.001$).

Figure 15: Capsaicin Dose-Response Curves with and without 18.8 mg/kg Capsazepine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 18.8 mg/kg capsazepine and the other five with intralipid vehicle. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was a statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p < 0.001$).

Sodium Metabisulfite Dose-Response Curves

Figure 16: Sodium Metabisulfite Dose-Response Curves with and without 10 mg/kg Morphine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 10 mg/kg morphine and the other five with saline. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was a statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p < 0.05$).

Figure 17: Sodium Metabisulfite Dose-Response Curves with and without 18.8 mg/kg Capsazepine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 18.8 mg/kg capsazepine and the other five with intralipid vehicle. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was no statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p > 0.05$).

Figure 18: Ammonia Dose-Response Curves with and without 10 mg/kg Morphine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 10 mg/kg morphine and the other five with saline. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was no statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p > 0.5$).

Figure 19: Ammonia Dose-Response Curves with and without 18.8 mg/kg Capsazepine. Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with 18.8 mg/kg capsazepine and the other five with intralipid vehicle. The experimenter was blinded as to which mouse got what. The experiment was performed on two groups of mice and the results were combined. Each point is the average TCAS score of ten mice ($\pm$ SEM). The EC50 values are means ($\pm$ SEM) of EC50s calculated for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. Using the Student's t Test, there was a statistically significant difference between the $\log_{10}$ EC50s of the two groups ($p < 0.001$).

Table 3: EC50s of Four Pulmonary Irritants, and their Modification by Morphine and Capsazepine.

	Citric Acid (mM)	Capsaicin (mM)	Sodium Metabisulfite (mM)	Ammonia (M)
Control	33.2 ± 10.2	0.34 ± 0.08	13.4 ± 3.5	0.16 ± 0.06
10 mg/kg Morphine	260 ± 90 (<i>p</i> <0.05)	6.1 ± 2.5 (<i>p</i> <0.001)	56.7 ± 16.2 (<i>p</i> <0.05)	0.18 ± 0.07 (<i>p</i> >0.5)
Control	20.9 ± 3.9	0.35 ± 0.09	9.5 ± 2.9	0.21 ± 0.06
18.8 mg/kg Capsazepine	70.6 ± 19.1 (<i>p</i> <0.05)	2.1 ± 0.4 (<i>p</i> <0.001)	31.5 ± 12.2 (<i>p</i> >0.05)	1.4 ± 0.3 (<i>p</i> <0.001)

Fifteen minutes prior to the start of the experiment, five of the ten mice to be tested together were injected (ip) with one of the antagonists and the other five with vehicle (saline for the morphine experiments and 20% intralipid emulsion for the capsazepine experiments). The experimenter was blinded as to which mouse got what. The experiment was performed twice and the results were combined (*n*=10). Every tabulated EC50 value is the mean (±SEM) of EC50s calculated for each individual mouse. The *p*-value from a Student's *t* Test of the log₁₀ EC50s is italicised.

3.3.4. *Reproducibility of the Results*

Table 4 contains EC50 values of four dose-response experiments with each of the four pulmonary irritants. In addition, it also contains the results from four dose-response experiments with each of the irritants, in which half the mice being tested together were control mice, while the rest were injected with one of the antagonists. In the latter category (where half the mice were injected with an antagonist and the other five were not), the EC50 values between the four groups for each irritant were similar to each other, as evidenced by the p-value from an analysis of variance of the $\log_{10}$ EC50s.

In the former category where all ten mice were without an antagonist, it was found that there was a difference in EC50s between the four groups when tested with capsaicin, sodium metabisulfite and ammonia; the EC50s of the four groups tested with citric acid showed no difference ($p>0.1$), however there was variation between them.

Furthermore, when comparing the mean EC50s between these two categories, it was found that for citric acid, capsaicin and sodium metabisulfite, the EC50s of the experiments where half the mice were injected with the antagonist, were significantly lower than the EC50s obtained when none of the mice were injected with the antagonist. There was no difference found between the EC50s with ammonia ($p>0.1$).

This difference could be explained with the "negative" group effect that was found from the correlation analysis (section 3.3.2, page 33). During the experimental run when half the mice were injected with control and the other half with either morphine or capsazepine, the antagonist mice did not feel as much discomfort as the saline mice. As a result, they avoided the irritant in the Test Chamber less, resulting in

Table 4: Differences in EC50s to Four Pulmonary Irritants with Either Half or All the Mice Without an Antagonist.

	5 Mice of 10 Without Antagonist		All 10 Mice Without Antagonist	
Citric Acid (mM)	45.2 ± 15.6	] (p>0.1)	27.4 ± 5.3	] (p>0.1)
	18.0 ± 12.4		46.3 ± 12.0	
	21.8 ± 7.6		46.6 ± 18.2	
	20.2 ± 3.1		65.8 ± 13.7	
Total Mean	26.8 ± 5.5		46.6 ± 6.7	p<0.05
Capsaicin (mM)	0.21 ± 0.11	] (p>0.1)	1.3 ± 0.3	] (p<0.05)
	0.47 ± 0.09		0.7 ± 0.2	
	0.33 ± 0.08		1.2 ± 0.4	
	0.37 ± 0.17		0.5 ± 0.1	
Total Mean	0.34 ± 0.06		0.9 ± 0.1	p<0.01
Sodium Metabisulfite (mM)	14.2 ± 5.6	] (p>0.1)	36.9 ± 5.8	] (p<0.05)
	12.8 ± 4.8		19.2 ± 3.5	
	5.6 ± 3.5		19.4 ± 4.6	
	14.8 ± 4.8		14.7 ± 3.2	
Total Mean	12.1 ± 2.4		22.5 ± 2.5	p<0.05
Ammonia (mM)	225.8 ± 115.5	] (p>0.5)	183.0 ± 32.4	] (p<0.05)
	90.8 ± 29.2		57.3 ± 20.3	
	128.6 ± 43.8		70.8 ± 13.3	
	295.6 ± 103.1		71.8 ± 22.8	
Total Mean	185.2 ± 41.8		95.1 ± 14.2	p>0.1

Dose-response curves to the irritants were obtained from four different groups of ten mice. Where there were five mice out of ten without antagonist (middle column), the remaining five mice were injected with morphine or capsazepine. In the right column, all ten mice were without the antagonist. Every tabulated EC50 is the mean (±SEM) of EC50s calculated for each individual mouse; the p-value from an Analysis of Variance of the log₁₀ EC50s is displayed immediately to the right of the EC50s of the four groups. The differences in total mean EC50 (bold) between the two columns, with their corresponding p-values from a Student's t Test of log₁₀ EC50s, may relate to avoidance of crowding ("negative" group effect).

some of the mice remaining inside it. Because mice tended to avoid each other, the control mice stayed away from the antagonist mice, even if the irritant was not noxious. With all ten mice tested together without an antagonist, all the mice felt discomfort due to the irritant at about the same time; thus, they avoided the Test Chamber only when the atmosphere became noxious. As a result, the mice had an apparent EC50 value that was higher compared to the group with only five control mice.

3.3.5. *Absence of Desensitisation*

These experiments were performed to determine whether desensitisation occurred when mice were exposed several times to dose-response curves with the four pulmonary irritants. Ten mice were re-tested with each of the irritants four times at 15-minute intervals.

Figures 20 through to 23 show the results of the above experiments. With citric acid (figure 20), the first dose-response curve resulted in an EC50 of 70.5 ± 17.1 mM. The subsequent re-tests had EC50s of 15.0 ± 7.2 mM, 6.8 ± 1.1 mM, and 2.4 ± 0.3 mM, respectively. For capsaicin (figure 21), the EC50 values were 1.24 ± 0.27 mM, 0.35 ± 0.11 mM, 45.5 ± 13.4 μ M, and 20.8 ± 4.7 μ M, respectively. For sodium metabisulfite (figure 22), the EC50s were 36.9 ± 5.8 mM, 26.2 ± 10.6 mM, 2.5 ± 0.3 mM, and 1.3 ± 0.3 mM, respectively. Finally, for ammonia (figure 23), the EC50s of the four dose-response curves were 183.0 ± 32.4 mM, 42.3 ± 11.0 mM, 37.3 ± 12.5 mM, and 4.0 ± 1.6 mM, respectively. The results indicate that the mice did not experience desensitisation, but rather sensitisation. This could mean that they either felt discomfort at lower aerosol concentrations, or they learned to avoid the irritant sooner. This suggests that the MASIAC could be used to evaluate learning curves in mice.

Citric Acid Dose-Response Curves - 4 Exposures

Figure 20: Four Exposures of Citric Acid Dose-Response Curves on the Same Group of Mice. Each point is the average TCAS score of ten mice ($\pm$ SEM). Each EC50 value is the mean ($\pm$ SEM) of EC50s for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. The dose-response curves were performed every 15 minutes on the same group of mice. The EC50s were 70.6 ± 17.2 mM, 15.0 ± 7.2 mM, 6.8 ± 1.2 mM, and 2.4 ± 0.3 mM, respectively.

Capsaicin Dose-Response Curves - 4 Exposures

Figure 21: Four Exposures of Capsaicin Dose-Response Curves on the Same Group of Mice. Each point is the average TCAS score of ten mice ($\pm$ SEM). Each EC50 value is the mean ($\pm$ SEM) of EC50s for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. The dose-response curves were performed every 15 minutes on the same group of mice. The EC50s were 1.2 ± 0.3 mM, 0.35 ± 0.11 mM, 45.5 ± 13.4 μ M, and 20.8 ± 4.7 μ M, respectively.

Sodium Metabisulfite Dose-Response Curves - 4 Exposures

Figure 22: Four Exposures of Sodium Metabisulfite Dose-Response Curves on the Same Group of Mice. Each point is the average TCAS score of ten mice ($\pm$ SEM). Each EC50 value is the mean ($\pm$ SEM) of EC50s for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. The dose-response curves were performed every 15 minutes on the same group of mice. The EC50s were 36.9 ± 5.8 mM, 26.6 ± 10.6 mM, 2.5 ± 0.3 mM, and 1.3 ± 0.3 mM, respectively.

Figure 23: Four Exposures of Ammonia Dose-Response Curves on the Same Group of Mice. Each point is the average TCAS score of ten mice ($\pm$ SEM). Each EC50 value is the mean ($\pm$ SEM) of EC50s for each individual mouse. The dotted line is the expected baseline score with no irritant. Dose is defined as the concentration of irritant in the nebulizer. The dose-response curves were performed every 15 minutes on the same group of mice. The EC50s were 183.0 ± 32.4 mM, 42.3 ± 11.0 mM, 37.3 ± 12.5 mM, and 4.0 ± 1.6 mM, respectively.

Following the multiple exposure experiment with citric acid (figure 20), those same mice were exposed to an additional dose-response experiment with citric acid the next day and three days after that. The EC50s from those experiments were 19.8 ± 7.0 mM and 63.4 ± 18.0 mM, respectively. This indicates that the mice were still sensitised to, or remembered, the citric acid one day after the multiple exposure experiment, but forgot it, or recovered from it by the fourth day. These follow-up experiments were only done with citric acid. Further research should be conducted in this area with a variety of pulmonary irritants to ascertain whether the mice are being sensitised to the irritant or are learning to avoid it, and to determine the feasibility of using the MASIAC as an additional tool in animal behavioural research.

4. DISCUSSION

4.1. MASIAC Chamber

The greatest disadvantage of currently used assays is that they trap the animals in irritating atmospheres without any possibility of escape (section 1.6, page 14). Our intent was to develop a new assay that would be as good as standard methods in evaluating pulmonary irritation, while minimising the stress that the animals perceive. For this reason, the MASIAC was developed based on the principle of avoidance, where the mice would have a chance to escape the irritant if it were unpleasant.

As stated in section 1.5 (page 10), there are several factors that have to be considered when using an exposure chamber:

4.1.1. Factors to Consider with Exposure Systems

4.1.1.1. Temperature and Humidity

The whole-body exposure system was chosen for this assay because it provided "real world" responses to irritants, in that the entire body was exposed to the noxious agent, and not just the head or nose. Also, there weren't any specific measures in place to control temperature and humidity inside the chamber; thus, the MASIAC was built as a dynamic exposure chamber. The delivery of fresh aerosol into the chamber and its removal allowed for maintenance of temperature and humidity at a constant level. In addition, because the mice were inside the MASIAC for no more than 5 minutes at a time, the change in both temperature and humidity was insignificant.

4.1.1.2. Uniform Distribution and Aerosol Deposition

Other factors to be considered include uniformity of aerosol distribution within the exposure chamber and deposition of the substance on the animals' fur. By observing the flow of the aerosol through the chambers, it looked as if it was uniformly distributed. However, the chamber atmosphere was not sampled at anytime during the experiments. It was also noted that the movement of mice disrupted the helical flow of the aerosol, but it was restored within a few seconds. The deposition of the aerosol on the animal fur and chamber walls was minimised by limiting the exposure time to only 5 minutes. Most current assays expose animals to the irritant for much longer periods of time, up to 24 hours (MacFarland, 1987).

4.1.1.3. Type of Nebulizer

The nebulizer used for these experiments was the VixOne nebulizer from Westmed Inc. (section 2.1, page 16). At an optimised flow rate of 5 L/min, it produces aerosol particles with a MMAD of 2.6 μm (obtained from the Westmed Inc. website – www.westmedinc.com). In a recent study in which a number of nebulizers were compared, the VixOne was one of the more efficient nebulizer systems (Hess *et al*, 1996). In addition, the VixOne nebulizer was used because it was easily obtainable.

4.1.2. Evaluation of a New Assay

Whenever a new assay is developed, it must be evaluated to determine how well it compares to other standard assays. The criteria used for evaluating new assays are well documented, and they include the accuracy and precision of the results, the

sensitivity and specificity of the new method, as well as the cost and time required to conduct the experiments (Baffi, 1997; Friedman and Wyatt, 1997).

4.1.2.1. Accuracy

The accuracy of results is defined as "the degree to which a measurement ... represents the true value of the attribute that is being measured" (Spraycar, 1995). It is not known what the true EC50 values are for irritation by the four irritants; however, an estimate of the accuracy of the results can be obtained by comparing them to results from standard assays. As already mentioned in section 3.2.2 (page 28), the citric acid EC50s were considerably lower than the concentrations commonly used by other standard assays, and the capsaicin EC50s were within the range of conventionally used concentrations. For sodium metabisulfite, the typically used concentrations ranged from 3 mM upto 0.8 M (Sakamoto *et al*, 1992; Yeo *et al*, 1992; Sakamoto *et al*, 1994; Atzori *et al*, 1997). The EC50s that were obtained with the MASIAC were around 30 to 50 mM, which lie in the lower area of the range. Ammonia is usually found to be noxious at a concentration of around 0.3 M (Matsumoto *et al*, 1994a; Matsumoto *et al*, 1994b); the MASIAC detected the irritation at slightly lower concentrations. This suggests that the MASIAC is an effective and reproducible assay.

4.1.2.2. Precision

Precision is defined as the "reproducibility of a quantifiable result" or "an indication of the random error" (Spraycar, 1995). By performing the same experiment on several groups of mice, some estimation of the reproducibility of results was obtained. Table 4 in section 3.3.4 (page 53) indicates that only EC50s of citric acid

dose-response curves were not significantly different from each other, even though there was some variation between the four groups. Table 4 also shows that there was a difference between EC50 values when all ten mice were tested without an antagonist as opposed to only five of them. This suggests that there may be a problem in using the MASIAC to evaluate agents that reduce the perceived irritation. Ironically, when half the mice were injected with the antagonist, the EC50s of the control groups were not significantly different from each other. More research should be conducted in this area to determine the reason for these differences and whether they are related to the tendency of the mice to avoid other mice (section 3.3.2, page 33).

Furthermore, in section 3.3.1.1 (page 33), the standard error range for a group of 10 mice was predicted to be within about 40% of the mean EC50. Table 4 indicates that the calculated error values were smaller (about 20% of the mean EC50). This discrepancy can be explained with the variation that was observed between the four groups for every irritant tested. When the EC50 values from all those groups were combined to examine the distribution of EC50 values (section 3.3.1.1, page 33), the resultant variance was exaggerated, thus the predicted error range for a group of 10 mice was also overestimated.

4.1.2.3.Sensitivity

Sensitivity can be defined as the degree of response to a change in concentration of the substance being tested. (Spraycar, 1995). The MASIAC is quite sensitive because the mice avoided the irritant in a dose-dependent fashion. This was observed with all four irritants. In addition, the EC50s that were calculated for citric acid and ammonia were lower than concentrations reported in the literature. This suggests

that the MASIAC is more sensitive than currently used methods because an animal's escape reaction can be expected to occur at lower concentrations than the physiological distress (change in respiratory rate and cough) that is commonly measured.

4.1.2.4. Specificity

Specificity can be defined as "freedom from interference by any element or compound other than the analyte" (Spraycar, 1995). In other words, how well can the assay detect the specific agent in question compared to other substances. This was not investigated; however, in theory, the MASIAC can detect any form of irritation or discomfort that the mice experience because they can avoid it as soon as they feel it. The experimenter, unfortunately, cannot distinguish between the various forms of irritation (nasal or pulmonary irritation, or smell).

The results confirm the above assumption because the MASIAC detected the irritation elicited by the four irritants. Therefore, the irritation that the MASIAC detects does not seem to be limited to any particular type of irritant.

4.1.2.5. Incidence of False Positive/Negative Results

The incidence of false positive and false negative results was not investigated, but their rate of appearance would be expected to be low. They would be observed only if something other than the irritant affected the movement of the mice. These could include a difference in temperature, relative humidity, aerosol flow rate or amount of noise between the chambers. In all experiments that were carried out, all the above factors were maintained at a constant level between the two chambers in order to

minimise those sources of variance. Furthermore, the incidence of false results was reduced by taking five measurements from each run instead of one, because the distribution of mice between the chambers at any one time may not have reflected their perceived irritation. By taking those five measurements, a mean with a variance of responses between the mice was obtained.

4.1.2.6. Cost and Time Required

These two factors do not have a direct effect on the results of a new assay; however, they do impact the experimenters that are using it. The cost of the MASIAC is substantially reduced compared to current assays. There is no need for any big stainless-steel exposure chambers or for any diagnostic equipment, such as body plethysmographs and recording microphones. The plexiglas chamber is easy to maintain; it does not require any special measures to clean it and it can be used for a long period of time. The amount of time that is required to conduct an experiment is also less. In just under two hours, a complete dose-response experiment can be performed.

4.1.2.7. Testing Groups of Mice Simultaneously

The results from the correlation analysis that was performed on the movement of the mice indicated that they did not have any tendency to cluster (section 3.3.2, page 33). In fact, the analysis indicated that the mice tended to avoid each other (the "negative" group effect). This was observed when all four irritants were tested on the mice, but not with distilled water or randomly generated avoidance scores. This indicates that it is valid to test groups of mice simultaneously.

Furthermore, the experiments where morphine and capsazepine were investigated on the dose-response curves to the four irritants, five of the ten mice that were tested together were injected with one of the antagonists. So in effect there were two different groups of mice being tested at the same time. The fact that these two groups could be distinguished provides further evidence that the group effect does not have a significant influence on the responses of the mice.

4.2. Pharmacology of the Respiratory Tract

Citric acid and capsaicin were used because they are standard commonly used pulmonary irritants. Sodium metabisulfite and ammonia are irritants that human beings are exposed to every day; sodium metabisulfite is used as a food and wine preservative (Atzori *et al*, 1997) and ammonia is used in household cleaners and detergents. It was for those reasons that these particular agents were used to evaluate the MASIAC.

For the antagonists, morphine was used because it is an opioid agonist and is commonly used as an analgesic, as well as antitussive; it has been shown that morphine can reduce the perceived irritation following inhalation of capsaicin (Fuller *et al*, 1988). Capsazepine was chosen because of its selectivity for the vanilloid (capsaicin) receptor (Bevan *et al*, 1992). Therefore, these two antagonists were used to determine if the MASIAC could detect a shift in the dose-response curves of the four pulmonary irritants.

4.2.1. Citric Acid and Capsaicin

Citric acid is believed to stimulate the unmyelinated *C fibre receptors* (Lalloo *et al*, 1995); however, there is evidence suggesting that it may also stimulate *rapidly*

adapting irritant receptors (Morice *et al*, 1992). Capsaicin, the active ingredient of hot peppers, is believed to be a selective agonist of the vanilloid receptors (Szallasi and Blumberg, 1996), which are associated with C fibre endings (Fuller, 1991; Wang *et al*, 1996). It has been hypothesised that the vanilloid receptor's endogenous ligand is the proton (Bevan and Geppetti 1994; Szallasi and Blumberg, 1996), which could explain why citric acid also acts upon C fibre endings. Mohammed and colleagues have suggested that inhaled capsaicin activates both *C fibre* and *rapidly adapting irritant receptors* (Mohammed *et al*, 1993), but Bergren has hypothesised that the activation of the *irritant receptors* was due to changes in lung mechanics as a result of *C fibre* stimulation (Bergren, 1997). The same hypothesis could explain why there is evidence that citric acid can stimulate both of these receptors.

Morphine, which produces analgesia by stimulating opioid receptors (Reisine and Pasternak, 1996), was able to reduce the perceived irritation as evidenced by the shift in the dose response curves to both citric acid and capsaicin. The same was observed with capsazepine, which is consistent with both irritants acting through a similar mechanism. However, because the shifts in the dose-response curves with capsazepine were not parallel, this suggests that there may be a site other than the *C fibre receptors* that mediate the noxious effects.

4.2.2. Sodium Metabisulfite

Sodium metabisulfite has an unclear mechanism of action. It is believed that an equilibrium exists where sodium metabisulfite is hydrolysed into sulfite and hydrogen ions (Sakamoto *et al*, 1994; Atzori *et al*, 1997); these protons then stimulate the *C fibre receptors* (Atzori *et al*, 1997). However the role of the sulfite ions is unknown. It has

been reported that the effects due to sodium metabisulfite stimulation are mediated through endogenously released tachykinins and that this release is mediated through a capsaicin-sensitive pathway (Sakamoto *et al*, 1992; Sakamoto *et al*, 1994), which suggests that *C fibre receptors* may be involved.

Results obtained with the MASIAC indicate that the irritation was perceived to be painful because morphine shifted the sodium metabisulfite dose-response curve. Capsazepine shifted the curve, but not to a significant degree (figure 17). This suggests that either the dose of capsazepine was too small, or, in addition to the *C fibre receptor*, sodium metabisulfite acts via an additional site where endogenous tachykinins are released.

4.2.3. Ammonia

Ammonia is a base (Coon *et al*, 1970), and is believed to elicit its effects selectively on *rapidly adapting irritant receptors* (Karlsson *et al*, 1988). Our results provide evidence to the contrary. If ammonia was selective for the *irritant receptor*, then capsazepine should not have had any effect. But, as figure 19 shows, capsazepine produced a significant shift in the ammonia dose-response curve. This suggests that ammonia has some action upon the C fibre endings in the respiratory tract.

Furthermore, morphine had no effect on the ammonia dose-response curve. This suggests that the stimulation by ammonia was not perceived as painful to the mice. From personal experience, ammonia is a very smelly substance. It is possible that the mice avoided ammonia because of the smell. Morphine does not affect an animal's sense of smell (Reisine and Pasternak, 1996); thus, it would not change the responses to a smelly substance such as ammonia.

4.3. Future Avenues for Research

4.3.1. Atmospheric and Environmental Sciences

The data presented above suggest the MASIAC is a sensitive assay at detecting irritating substances. This feature can be used as a quick and easy way to evaluate pollutants in the atmosphere. The results may provide the user with a general idea of the pollutant's effects on an animal's pulmonary system, and a "ball-park" figure for a concentration of the pollutant that is perceived as noxious.

4.3.2. Animal Behavioural Research

From experiments where dose-response curves were performed on the same group of mice (section 3.3.5, page 54), the mice either learned to avoid the irritant and/or were sensitised to it. If in fact the mice were learning to avoid the irritant, then this finding suggests the use of the MASIAC as a tool to evaluate learning curves. Investigations could be carried out to determine whether or not the mice would "forget" the uncomfortable effects of the irritant, for example following administration of benzodiazepine sedatives, which cause amnesia at sub-anaesthetic doses (Voigt *et al*, 1996). Such experiments could provide information on the feasibility of using the MASIAC as an additional tool in animal behavioural research.

4.3.3. Asthma

People who suffer from asthma are very sensitive to particular agents such as histamine, more so than normal people (Spina and Page, 1996). If the shift in the dose response curves that was observed following multiple exposures with the four irritants (section 3.3.5, page 54) was not due to the mice learning to avoid the irritant but rather

sensitisation, then those mice could be used to represent an animal model of asthmatics and the MASIAC could potentially be used to evaluate new agents in the treatment of this condition. But first, more research must be conducted to determine conclusively if the mice were sensitised to the irritant, or were they learning to avoid it.

4.4. Conclusion

To minimise the physical and psychological stress that animals experience in currently used methods, a new assay to evaluate pulmonary irritants was developed based on the principle of avoidance. The Minimal Animal Stress Irritant Assay Chamber (MASIAC) was evaluated and found to be effective, reproducible and sensitive. In addition, the assay is not limited to any particular type of irritant, because the MASIAC has the potential to detect any form of discomfort that the mice perceive. Nevertheless, there are a number of possible avenues for future research for the MASIAC, including environmental pollution, human asthma, and as a quantifiable model of learning.

5. BIBLIOGRAPHY

- Achmadi, U.F. & Pauluhn, J. (1998) Household insecticides: evaluation and assessment of inhalation toxicity: a workshop summary. *Experimental & Toxicologic Pathology*, **50**, 67-72.
- Acs, G., Lee, J., Marquez, V.E. & Blumberg, P.M. (1996) Distinct structure-activity relations for stimulation of ⁴⁵Ca uptake and for high affinity binding in cultured rat dorsal root ganglion neurons and dorsal root ganglion membranes. *Brain Research, Molecular Brain Research*, **35**, 173-182.
- Alarie, Y. (1973) Sensory irritation of the upper airways by airborne chemicals. *Toxicology & Applied Pharmacology*, **24**, 279-297.
- Alarie, Y. (1981) Bioassay for evaluating the potency of airborne sensory irritants and predicting acceptable levels of exposure in man. *Food & Cosmetics Toxicology*, **19**, 623-626.
- Atzori, L., Bannenberg, G., Corrigan, A.M., Congiu, L., Lundberg, J.M., Moldeus, P. & Ryrfeldt, A. (1997) Sodium metabisulfite and citric acid induce bronchoconstriction via a sulfite-sensitive pathway in the isolated guinea pig lung. *Respiration*, **64**, 145-151.
- Baffi, R.A. (1997) The role of assay validation in specification development. *Developments in Biological Standardizations*, **91**, 105-113.
- Barrow, C.S., Alarie, Y., Warrick, J.C. & Stock, M.F. (1977) Comparison of the sensory irritation response in mice to chlorine and hydrogen chloride. *Archives of Environmental Health*, **32**, 68-76.
- Barrow, C.S., Alarie, Y. & Stock, M.F. (1978) Sensory irritation and incapacitation evoked by thermal decomposition products of polymers and comparisons with known sensory irritants. *Archives of Environmental Health*, **33**, 79-88.
- Bergren, D.R. (1997) Sensory receptor activation by mediators of defense reflexes in guinea-pig lungs. *Respiration Physiology*, **108**, 195-204.
- Bevan, S., Hothi, S., Hughes, G., James, I.F., Rang, H.P., Shah, K., Walpole, C.S. & Yeats, J.C. (1992) Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *British Journal of Pharmacology*, **107**, 544-552.
- Bevan, S. & Geppetti, P. (1994) Protons: small stimulants of capsaicin-sensitive sensory nerves. *Trends in Neurosciences*, **17**, 509-512.
- Bickerman, H., Barach, A., Itkin, S. & Drimmer, F. (1954) The Experimental Production of Cough in Human Subjects Induced by Citric Acid Aerosols. Preliminary Studies on the Evaluation of Antitussive Agents. *American Journal of Medical Sciences*, 156-163.

- Blom, H.M., Van Rijswijk, J.B., Garrelds, I.M., Mulder, P.G., Timmermans, T., Gerth & van Wijk, R. (1997) Intranasal capsaicin is efficacious in non-allergic, non-infectious perennial rhinitis. A placebo-controlled study. *Clinical & Experimental Allergy*, **27**, 796-801.
- Bolser, D.C., DeGennaro, F.C., O'Reilly, S., Hey, J.A. & Chapman, R.W. (1995) Pharmacological studies of allergic cough in the guinea pig. *European Journal of Pharmacology*, **277**, 159-164.
- Bolser, D.C., Del Prado, M., O'Reilly, S., Mingo, G. & Hey, J.A. (1995) Evan's blue dye blocks capsaicin-induced cough and bronchospasm in the guinea pig. *European Journal of Pharmacology*, **276**, R1-3.
- Bootle, D.J., Adcock, J.J. & Ramage, A.G. (1996) Involvement of central 5-HT_{1A} receptors in the reflex activation of pulmonary vagal motoneurons by inhaled capsaicin in anaesthetized cats. *British Journal of Pharmacology*, **117**, 724-728.
- Bootle, D.J., Adcock, J.J. & Ramage, A.G. (1998) The role of central 5-HT receptors in the bronchoconstriction evoked by inhaled capsaicin in anaesthetised guinea-pigs. *Neuropharmacology*, **37**, 243-250.
- Bourin, M., Hascoet, M., Mansouri, B., Colombel, M.C. & Bradwejn, J. (1992) Comparison of behavioral effects after single and repeated administrations of four benzodiazepines in three mice behavioral models. *Journal of Psychiatry & Neuroscience*, **17**, 72-77.
- Buff, R. & Koller, E.A. (1974) Studies on mechanisms underlying the reflex hyperpnoea induced by inhalation of chemical irritants. *Respiration Physiology*, **21**, 371-383.
- Cahill, L., Brioni, J. & Izquierdo, I. (1986) Retrograde memory enhancement by diazepam: its relation to anterograde amnesia, and some clinical implications. *Psychopharmacology*, **90**, 554-556.
- Chan-Yeung, M., Kinsella, M. & Ostrow, D.N. (1990) Specific bronchoprovocation testing. *Clinical Reviews in Allergy*, **8**, 147-157.
- Chou, Y.C., Liao, J.F., Chang, W.Y., Lin, M.F. & Chen, C.F. (1999) Binding of dimemorfan to sigma-1 receptor and its anticonvulsant and locomotor effects in mice, compared with dextromethorphan and dextrorphan. *Brain Research*, **821**, 516-519.
- Cheng, Y.S. & Moss, O.R. (1995) Inhalation exposure systems. In: Concepts in Inhalation Toxicology, 2nd edition, McClellan, R.O. & Henderson, R.F., editors, Taylor & Francis, Washington, DC, USA, p 25-66.
- Clarke, R.S. (1983) New drugs--boon or bane? Premedication and intravenous induction agents. *Canadian Anaesthetists Society Journal*, **30**, 166-173.

- Cliff, M.A. & Green, B.G. (1996) Sensitization and desensitization to capsaicin and menthol in the oral cavity: interactions and individual differences. *Physiology & Behavior*, **59**, 487-494.
- Conley, J., Yang, H., Wilson, T., Blasetti, K., Di Ninno, V., Schnell, G. & Wong, J.P. (1997) Aerosol delivery of liposome-encapsulated ciprofloxacin: aerosol characterization and efficacy against *Francisella tularensis* infection in mice. *Antimicrobial Agents & Chemotherapy*, **41**, 1288-1292.
- Coon, R.A., Jones, R.A., Jenkins, L.J.J. & Siegel, J. (1970) Animal inhalation studies on ammonia, ethylene glycol, formaldehyde, dimethylamine, and ethanol. *Toxicology & Applied Pharmacology*, **16**, 646-655.
- Covert, D.S. & Frank, N.R. (1980) Atmospheric particles: behavior and functional effects. In: *Physiology and Pharmacology of the Airways*, Nadel, J.A., editor, Marcel Dekker Inc, New York, USA, p 259-295.
- Dambisya, Y.M., Chan, K. & Wong, C.L. (1992) Dispositional study of opioids in mice pretreated with sympathomimetic agents. *Journal of Pharmacy & Pharmacology*, **44**, 687-690.
- Dicpinigaitis, P.V. & Rauf, K. (1998) The influence of gender on cough reflex sensitivity. *Chest*, **113**, 1319-1321.
- Doherty, M.J., Wang, L.J., Donague, S., Pearson, M.G., Downs, P., Stoneman, S.A., Earis & JE. (1997) The acoustic properties of capsaicin-induced cough in healthy subjects. *European Respiratory Journal*, **10**, 202-207.
- Dorato, M.A. & Wolff, R.K. (1991) Inhalation exposure technology, dosimetry, and regulatory issues. *Toxicologic Pathology*, **19**, 373-383.
- Dray, A. (1992) Neuropharmacological mechanisms of capsaicin and related substances. *Biochemical Pharmacology*, **44**, 611-615.
- Dray, A. (1992) Mechanism of action of capsaicin-like molecules on sensory neurons. *Life Sciences*, **51**, 1759-1765.
- Ellis, J.L., Sham, J.S. & Undem, B.J. (1997) Tachykinin-independent effects of capsaicin on smooth muscle in human isolated bronchi. *American Journal of Respiratory & Critical Care Medicine*, **155**, 751-755.
- Featherstone, R.L., Parry, J.E., Evans, D.M., Jones, D.M., Olsson, H., Szelke, M. & Church, M.K. (1996) Mechanism of irritant-induced cough: studies with a kinin antagonist and a kallikrein inhibitor. *Lung*, **174**, 269-275.
- Field, P.I., McClean, M., Simmul, R. & Berend, N. (1994) Comparison of sulphur dioxide and metabisulphite airway reactivity in subjects with asthma. *Thorax*, **49**, 250-256.

- File, S.E. (1985) What can be learned from the effects of benzodiazepines on exploratory behavior?. *Neuroscience & Biobehavioral Reviews*, **9**, 45-54.
- Fishman, E.G., Jenkins, L.J.J., Coon, R.A. & Jones, R.A. (1969) Effects of acute and repeated inhalation of dimethyl sulfoxide in rats. *Toxicology & Applied Pharmacology*, **15**, 74-82.
- Fossati, A., Vimercati, M.G., Caputo, R. & Valenti, M. (1995) Pharmacological profile of dextrophan. *Arzneimittel-Forschung*, **45**, 1188-1193.
- Friedman, C.P., and Wyatt, J.C. (1997) Evaluation Methods in Medical Informatics, Springer-Verlag, New York, USA, p .
- Fuller, R.W., Karlsson, J.A., Choudry, N.B. & Pride, N.B. (1988) Effect of inhaled and systemic opiates on responses to inhaled capsaicin in humans. *Journal of Applied Physiology*, **65** , 1125-1130.
- Fuller, R.W. (1991) Pharmacology of inhaled capsaicin in humans. *Respiratory Medicine*, **85 Suppl A**, 31-34.
- Fusco, B.M. & Giacobazzo, M. (1997) Peppers and pain. The promise of capsaicin. *Drugs*, **53**, 909-914.
- Gagnaire, F., Azim, S., Bonnet, P., Hecht, G. & Hery, M. (1994) Comparison of the sensory irritation response in mice to chlorine and nitrogen trichloride. *Journal of Applied Toxicology*, **14**, 405-409.
- Ganong, W.F. (1995) Review of Medical Physiology, 17th edition, Appleton & Lange, Norwalk, Connecticut, USA, p 591-624.
- Gearhart, J.M. & Schlesinger, R.B. (1988) Response of the tracheobronchial mucociliary clearance system to repeated irritant exposure: effect of sulfuric acid mist on function and structure. *Experimental Lung Research*, **14**, 587-605.
- Greenblatt, D.J., Ehrenberg, B.L., Gunderman, J., Scavone, J.M., Tai, N.T., Harmatz, JS & Shader, R.I. (1989) Kinetic and dynamic study of intravenous lorazepam: comparison with intravenous diazepam. *Journal of Pharmacology & Experimental Therapeutics*, **250**, 134-140.
- Greenblatt, D.J. & Sethy, V.H. (1990) Benzodiazepine concentrations in brain directly reflect receptor occupancy: studies of diazepam, lorazepam, and oxazepam. *Psychopharmacology*, **102**, 373-378.
- Gunnison, A.F., Sellakumar, A., Currie, D. & Snyder, E.A. (1987) Distribution, metabolism and toxicity of inhaled sulfur dioxide and endogenously generated sulfite in the respiratory tract of normal and sulfite oxidase-deficient rats. *Journal of Toxicology & Environmental Health*, **21**, 141-162.

- Halldorsdottir, H., Greiff, L., Wollmer, P., Andersson, M., Svensson, C., Alkner, U & Persson, C.G. (1997) Effects of inhaled histamine, methacholine and capsaicin on sputum levels of alpha 2-macroglobulin. *Thorax*, **52**, 964-968.
- Hansson, L., Midgren, B. & Karlsson, J.A. (1994) Effects of inhaled lignocaine and adrenaline on capsaicin-induced cough in humans. *Thorax*, **49**, 1166-1168.
- Hess, D., Ficher, D., Williams, P., Pooler, S. & Kacmarek, P.M. (1996) Medication nebulizer performance: effects of diluent volume, nebulizer flow, and nebulizer brand. *Chest*, **110**, 498-505.
- Hodgson, A., Moody, J.C., Stradling, G.N., Bailey, M.R. & Stather, J.W. (1989) A new small animal inhalation facility: design and performance. *Experimental Pathology*, **37**, 72-75.
- Hoel, D.G. (1979) Statistical approaches to toxicological data. *Environmental Health Perspectives*, **32**, 267-271.
- Hollander, W. (1988) Exposure facilities and aerosol generation and characterisation for inhalation experiments In: Inhalation Toxicology: The Design and Interpretation of Inhalation Studies and their Use in Risk Assessment, Mohr, U., editor; Springer-Verlag, New York, USA, p 67-85.
- Hoymann, H.G. & Heinrich, U. (1998) Measurement of lung function in rodents *in vivo*. In: Methods in Pulmonary Research, Uhlig, S. & Taylor, A.E., editors; Birkhauser-Verlag, Basel, Switzerland, p 1-28.
- Jamieson, D.M., Guill, M.F., Wray, B.B. & May, J.R. (1985) Metabisulfite sensitivity: case report and literature review. *Annals of Allergy*, **54**, 115-121.
- Joos, G.F., Germonpre, P.R. & Pauwels, R.A. (1995) Neurogenic inflammation in human airways: is it important? *Thorax*, **50**, 217-219.
- Kamei, J., Iwamoto, Y., Misawa, M. & Kasuya, Y. (1993) Effects of rimcazole, a specific antagonist of sigma sites, on the antitussive effects of non-narcotic antitussive drugs. *European Journal of Pharmacology*, **242**, 209-211.
- Karlsson, J.A., Sant'Ambrogio, G. & Widdicombe, J. (1988) Afferent neural pathways in cough and reflex bronchoconstriction. *Journal of Applied Physiology*, **65**, 1007-1023.
- Karrer, T. & Bartoshuk, L. (1995) Effects of capsaicin desensitization on taste in humans. *Physiology & Behavior*, **57**, 421-429.
- Kinnman, E., Nygard, E.B. & Hansson, P. (1997) Effects of dextromethorphan in clinical doses on capsaicin-induced ongoing pain and mechanical hypersensitivity. *Journal of Pain & Symptom Management*, **14**, 195-201.

- Kou, Y.R., Wang, C.Y. & Lai, C.J. (1995) Role of vagal afferents in the acute ventilatory responses to inhaled wood smoke in rats. *Journal of Applied Physiology*, **78**, 2070-2078.
- Lalloo, U.G., Fox, A.J., Belvisi, M.G., Chung, K.F. & Barnes, P.J. (1995) Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *Journal of Applied Physiology*, **79**, 1082-1087.
- Levitzky, M.G. (1999) Pulmonary Physiology, McGraw-Hill, New York, USA, p 189-229.
- Lewis, T.R., Morrow, P.E., McClellan, R.O., Raabe, O.G., Kennedy, G.L., Schwetz, B.A., Goehl, T.J., Roycroft, J.H. & Chhabra, R.S. (1989) Establishing aerosol exposure concentrations for inhalation toxicity studies. *Toxicology & Applied Pharmacology*, **99**, 377-383.
- MacFarland, H.N. (1983) Designs and operational characteristics of inhalation exposure equipment--a review. *Fundamental & Applied Toxicology*, **3**, 603-613.
- MacFarland, H.N. (1987) Designs and operational characteristics of inhalation exposure equipment. In: Inhalation Toxicology: Research Methods, Applications, and Evaluation, Salem, H., editor; Marcel Dekker, New York, USA, p 93-120.
- Marple, V.A. & Rubow, K.L. (1979) Aerosol generation concepts and parameters. In: Generation of Aerosols and Facilities for Exposure Experiments, Willeke, K., editor; Ann Arbor Science, Ann Arbor, MI, USA, p 3-29.
- Mathison, D.A., Stevenson, D.D. & Simon, R.A. (1985) Precipitating factors in asthma. Aspirin, sulfites, and other drugs and chemicals. *Chest*, **87**, 50S-54S.
- Matsumoto, S., Kanno, T., Nagayama, T., Yamasaki, M. & Shimizu, T. (1994a) Effects of veratridine and nifedipine on ammonia-induced rapidly adapting pulmonary stretch receptor stimulation in vagotomised rabbits. *Journal of the Autonomic Nervous System*, **48**, 133-142.
- Matsumoto, S., Yamasaki, M., Kanno, T., Nagayama, T., Tanno, M. & Shimizu, T. (1994b) Substance P antagonist does not block the stimulation of rapidly adapting pulmonary stretch receptors by ammonia. *Lung*, **172**, 31-45.
- Midgren, B., Hansson, L., Karlsson, J.A., Simonsson, B.G. & Persson, C.G. (1992) Capsaicin-induced cough in humans. *American Review of Respiratory Disease*, **146**, 347-351.
- Mohammed, S.P., Higenbottam, T.W. & Adcock, J.J. (1993) Effects of aerosol-applied capsaicin, histamine and prostaglandin E2 on airway sensory receptors of anaesthetized cats. *Journal of Physiology*, **469**, 51-66.
- Morice, A.H., Higgins, K.S. & Yeo, W.W. (1992) Adaptation of cough reflex with different types of stimulation. *European Respiratory Journal*, **5**, 841-847.

- Morikawa, T., Gallico, L. & Widdicombe, J. (1997) Actions of moguisteine on cough and pulmonary rapidly adapting receptor activity in the guinea pig. *Pharmacological Research*, **35**, 113-118.
- Morrow, P.E. & Mermelstein, R. (1988) Chronic inhalation toxicity studies: protocols and pitfalls. In: Inhalation Toxicology: The Design and Interpretation of Inhalation Studies and their Use in Risk Assessment, Mohr, U., editor; Springer-Verlag, New York, USA, p 103-117.
- Nadel, J.A. (1980) Autonomic regulation of airway smooth muscle. In: Physiology and Pharmacology of the Airways, Nadel, J.A., editor; Marcel Dekker Inc, New York, USA, p 217-257.
- Newman, S.P. (1993) Delivery of drugs to the respiratory tract. In: Pharmacology of the Respiratory Tract: Experimental and Clinical Research, Chung, K.F. & Barnes, P.J., editors; Marcel Dekker Inc, New York, USA, p 701-728.
- Nichol, G.M., Parsons, G.H. & Chung, K.F. (1994) Effect of sodium metabisulphite on bronchial blood flow in conscious sheep: pharmacological modulation. *British Journal of Pharmacology*, **111**, 918-922.
- Nicklas, R.A. (1989) Sulfites: a review with emphasis on biochemistry and clinical application. *Allergy Proceedings*, **10**, 349-356.
- O'Neill, T.P. (1991) Mechanism of capsaicin action: recent learnings. *Respiratory Medicine*, **85 Suppl A**, 35-41.
- Palecek, F., Sant'Ambrogio, G., Sant'Ambrogio, F.B. & Mathew, O.P. (1989) Reflex responses to capsaicin: intravenous, aerosol, and intratracheal administration. *Journal of Applied Physiology*, **67**, 1428-1437.
- Pauluhn, J. (1988) Different methods used in acute and subchronic inhalation studies of potential lung irritants, with particular attention to lung function measurements. In: Inhalation Toxicology: The Design and Interpretation of Inhalation Studies and their Use in Risk Assessment, Mohr, U., editor; Springer-Verlag, New York, USA, p 87-101.
- Perkins, M.N. & Campbell, E.A. (1992) Capsazepine reversal of the antinociceptive action of capsaicin in vivo. *British Journal of Pharmacology*, **107**, 329-333.
- Phalen, R.F., Mannix, R.C. & Drew, R.T. (1984) Inhalation exposure methodology. *Environmental Health Perspectives*, **56**, 23-34.
- Phalen, R.F., Yeh, H.C. & Prasad, S.B. (1995) Morphology of the respiratory tract. In: Concepts in Inhalation Toxicology, 2nd edition, McClellan, R.O. & Henderson, R.F., editors, Taylor & Francis, Washington, DC, USA, p 129-149.

- Philip, G., Sanico, A.M. & Togias, A. (1996) Inflammatory cellular influx follows capsaicin nasal challenge. *American Journal of Respiratory & Critical Care Medicine*, **153**, 1222-1229.
- Pinto, A., Yanai, M., Sekizawa, K., Aikawa, T. & Sasaki, H. (1995) Conditioned enhancement of cough response in awake guinea pigs. *International Archives of Allergy & Immunology*, **108**, 95-98.
- Pon, D.J., van Staden, C.J. & Rodger, I.W. (1994) Hypertrophic and hyperplastic changes of mucus-secreting epithelial cells in rat airways: assessment using a novel, rapid, and simple technique. *American Journal of Respiratory Cell & Molecular Biology*, **10**, 625-634.
- Pounsford, J.C. & Saunders, K.B. (1985) Diurnal variation and adaptation of the cough response to citric acid in normal subjects. *Thorax*, **40**, 657-661.
- Rang, H.P., Bevan, S. & Dray, A. (1991) Chemical activation of nociceptive peripheral neurones. *British Medical Bulletin*, **47**, 534-548.
- Ravi, K. & Singh, M. (1996) Role of vagal lung C-fibres in the cardiorespiratory effects of capsaicin in monkeys. *Respiration Physiology*, **106**, 137-151.
- Reisine, T. & Pasternak, G. (1996) Opioid analgesics and antagonists. In: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition, Hardman, J.G. & Limbird, L.E., editors; McGraw-Hill, p 521-555.
- Roche, N., Lurie, A., Authier, S. & Dusser, D.J. (1995) Nasal response to capsaicin in patients with allergic rhinitis and in healthy volunteers: effect of colchicine. *American Journal of Respiratory & Critical Care Medicine*, **151**, 1151-1158.
- Sakamoto, T., Elwood, W., Barnes, P.J. & Chung, K.F. (1992) Pharmacological modulation of inhaled sodium metabisulphite-induced airway microvascular leakage and bronchoconstriction in the guinea-pig. *British Journal of Pharmacology*, **107**, 481-487.
- Sakamoto, T., Tsukagoshi, H., Barnes, P.J. & Chung, K.F. (1994) Involvement of tachykinin receptors (NK1 and NK2) in sodium metabisulfite-induced airway effects. *American Journal of Respiratory & Critical Care Medicine*, **149**, 387-391.
- Salem, H. (1987) Factors influencing toxicity. In: Inhalation Toxicology: Research Methods, Applications, and Evaluation, Salem, H., editor; Marcel Dekker, New York, USA, p 35-57.
- Santos, A.R. & Calixto, J.B. (1997) Ruthenium red and capsazepine antinociceptive effect in formalin and capsaicin models of pain in mice. *Neuroscience Letters*, **235**, 73-76.

- Schaper, M. & Detwiler, K. (1991) Evaluation of the acute respiratory effects of aerosolized machining fluids in mice. *Fundamental & Applied Toxicology*, **16**, 309-319.
- Shuster, S. (1995) Capsaicin and the cause of causalgia. *Lancet*, **345**, 160-161.
- Silver, S.D. (1946) Constant flow gassing chambers: principles influencing design and operation. *Journal of Laboratory and Clinical Medicine*, **31**, 1153-1161.
- Simon, R.A. (1989) Sulfite challenge for the diagnosis of sensitivity. *Allergy Proceedings*, **10**, 357-362.
- Smaldone, G.C. (1996) Aerosolized drug delivery in the 90s. *Chest*, **110**, 316-317.
- Sotzing, J.H. & Brown, T.S. (1976) Chronic intermittent ethyl alcohol inhalation and avoidance learning. *Pharmacology, Biochemistry & Behavior*, **5**, 417-421.
- Spina, D. (1996) Airway sensory nerves: a burning issue in asthma? *Thorax*, **51**, 335-337.
- Spina, D. & Page, C.P. (1996) Airway sensory nerves in asthma--targets for therapy?. *Pulmonary Pharmacology*, **9**, 1-18.
- Spraycar, M., editor. (1995) Stedman's Medical Dictionary, 26th edition; Williams & Wilkins, Baltimore, MD, USA. p 10, 894, 1419, 1464, 1532, 1533, 1596, 1597, 1598, 1686, and 1927.
- Szallasi, A. & Blumberg, P.M. (1996) Vanilloid receptors: new insights enhance potential as a therapeutic target. *Pain*, **68**, 195-208.
- Takahama, K., Wakuda, I., Fukushima, H., Isohama, Y., Kai, H. & Miyata, T. (1997) Differential effect of codeine on coughs caused by mechanical stimulation of two different sites in the airway of guinea pigs. *European Journal of Pharmacology*, **329**, 93-97.
- Til, H.P. & Feron, V.J. (1992) Toxicology of sulphiting agents. I: Animal studies. *Food Additives & Contaminants*, **9**, 587-595.
- Ulrich, C.E., Haddock, M.P. & Alarie, Y. (1972) Airborne chemical irritants. Role of the trigeminal nerve. *Archives of Environmental Health*, **24**, 37-42.
- Vijayaraghavan, R., Schaper, M., Thompson, R., Stock, M.F. & Alarie, Y. (1993) Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract. *Archives of Toxicology*, **67**, 478-490.
- Voigt, J.P. & Morgenstern, E. (1992) Comparative effects of carbamazepine, phenytoin, diazepam and clonazepam on inhibitory avoidance learning in mice. *Psychopharmacology*, **108**, 131-135.

- Voigt, J.P., Morgenstern, E. & Fink, H. (1996) Memory-enhancing effects of benzodiazepines in mice. *European Neuropsychopharmacology*, **6**, 253-255.
- Wang, A.L., Blackford, T.L. & Lee, L.Y. (1996) Vagal bronchopulmonary C-fibers and acute ventilatory response to inhaled irritants. *Respiration Physiology*, **104**, 231-239.
- Weyel, D.A., Rodney, B.S. & Alarie, Y. (1982) Sensory irritation, pulmonary irritation, and acute lethality of a polymeric isocyanate and sensory irritation of 2,6-toluene diisocyanate. *Toxicology & Applied Pharmacology*, **64**, 423-430.
- Widdicombe, J. (1996) Sensory mechanisms. *Pulmonary Pharmacology*, **9**, 383-387.
- Widdicombe, J. & Sant' Ambrogio, G. (1996) Airway and pulmonary afferents and reflexes: a historical survey. In: Respiratory Physiology: People and Ideas; West, J.B., editor; Oxford University Press, p 319-351.
- Widdicombe, J.G. (1977) Defensive mechanisms of the respiratory system. *International Review of Physiology*, **14**, 291-315.
- Widdicombe, J.G. & Glogowska, M. (1973) Relative roles of irritant, type-J and pulmonary stretch receptors in lung reflexes. *Acta Neurobiologiae Experimentalis*, **33**, 21-31.
- Winter, J., Bevan, S. & Campbell, E.A. (1995) Capsaicin and pain mechanisms. *British Journal of Anaesthesia*, **75**, 157-168.
- Wright, C.E., Laude, E.A., Grattan, T.J. & Morice, A.H. (1997) Capsaicin and neurokinin A-induced bronchoconstriction in the anaesthetised guinea-pig: evidence for a direct action of menthol on isolated bronchial smooth muscle. *British Journal of Pharmacology*, **121**, 1645-1650.
- Yeo, C.T., O'Connor, B.J., Chen-Worsdell, M., Barnes, P.J. & Chung, K.F. (1992) Protective effect of loop diuretics, piretanide and frusemide, against sodium metabisulphite-induced bronchoconstriction in asthma. *European Respiratory Journal*, **5**, 1184-1188.
- Yeo, W.W., Higgins, K.S., Foster, G., Jackson, P.R. & Ramsay, L.E. (1995) Effect of dose adjustment on enalapril-induced cough and the response to inhaled capsaicin. *British Journal of Clinical Pharmacology*, **39**, 271-276.
- Zelenak, J.P., Alarie, Y. & Weyel, D.A. (1982) Assessment of the cough reflex caused by inhalation of sodium lauryl sulfate and citric acid aerosols. *Fundamental & Applied Toxicology*, **2**, 177-180.