Contains No CBI

American Chemistry Council

July 13, 2006

VIA HAND DELIVERY

mr# 297086

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
1201 Constitution Ave NW
Washington DC 20004-3302

8EHQ-05-16202

Attention: TSCA 8(e) Coordinator

RE: Supplemental Information on Submission Number 8EHQ-05-16202

Dear TSCA 8(e) Coordinator:

The American Chemistry Council's Diisocyanates Panel (Panel), on behalf of its members,[1] is submitting the attached final report, "MDI lung sensitization following topical induction: investigation of induction dose-response," to the EPA pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA). This is a follow-up to the supplemental submission of February 16, 2006 and original submission of September 9, 2005.

While being submitted in accordance with TSCA 8(e), the Panel has made no determination as to whether a substantial risk of injury to health or the environment is actually presented by this additional finding.

If you have any questions, please contact me, the Diisocyanates Panel Manager, at 703-741-5607 or sarah_mclallen@americanchemistry.com.

Best regards,

Sarah Loftus McLallen
Manager, Diisocyanates Panel

8906000038

Cc: DII Panel

---

[1] The members of the Panel are BASF Corporation, Bayer MaterialScience, The Dow Chemical Company, and Huntsman Polyurethanes.

III Project 256

III Report 11521

mx# 297086

MDI lung sensitisation following topical induction: investigation of induction dose-response

RECEIVED
06 JUL 13 AM 11:27

J Pauluhn

Bayer Healthcare AG
Wuppertal
Germany

The full report comprises 341 pages. This text does not include pages 43 to 341 (the Appendix), which contains detailed information on the study. The Appendix is available from the III Scientific Office on request.

Issued: June 2006

Number of pages: 42

Copyright International Isocyanate Institute Inc. 2006

Contains No CBI

III Report

International Isocyanate Institute Inc.

The Scientific Office, Bridgewater House, Whitworth Street, Manchester M1 6LT, UK.

The Scientific Office of the International Isocyanate Institute Inc. is operated by Gilbert International Limited, an independent contractor.

This report is on research sponsored by the International Isocyanate Institute, Inc.

The information, analysis, methods and recommendations herein are presented in good faith, are believed to be accurate and reliable, but may well be incomplete and/or not applicable to all conditions or situations that may be encountered.

No representation, guarantee or warranty is made as to the accuracy, reliability or completeness of this report, or that the application or use of any of the information, analysis, methods and recommendations herein will avoid, reduce or ameliorate hazards, accidents, losses, damages or injury of any kind to persons or property. Readers are therefore cautioned to satisfy themselves as to the applicability and suitability of said information, analysis, methods and recommendations for the purposes intended prior to use.

Bayer HealthCare

Report No.: AT02997

Date: May 15,2006

DIPHENYLMETHANE-4,4'-DIISOCYANATE
(polymeric MDI)

Lung Sensitization Study in Brown-Norway Rats following Topical Induction

STUDY DIRECTOR

Prof. Jürgen Pauluhn Ph.D., D.A.B.T.

SPONSOR

INTERNATIONAL ISOCYANATE INSTITUTE
2805 East Dupont Road
Ft. Wayne, IN 46845
U.S.A.

PERFORMING LABORATORY

BAYER HEALTHCARE AG
PH-GDD T MST
D-42096 Wuppertal
Germany

Bayer Project-no.: T4075868 / III-Proj. 256-EU-MTX

Study Completion Date: May 04, 2006

This page is intentionally left blank for the purpose of submitting administrative information that is required by regulations promulgated by various countries.

GLP COMPLIANCE STATEMENT

The study was conducted in compliance with the principles of Good Laboratory Practice described in the following issues:

- OECD Principles of Good Laboratory Practice (as revised in 1997) [ENV/MC/CHEM(98)17].
- Bulletin of the revised form of the chemicals act of June 20, 2002 Federal Law Gazette Volume 2002 (Part I No. 40, section 6, §19, issued at Bonn June 27, 2002).
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their application for tests on chemical substances (codified version), Official Journal of the European Union L50, February 20, 2004.
- EPA (Environmental Protection Agency) 40 CFR part 762 TSCA Good Laboratory Practice Standards.

As a deviation from these principles, this report was not audited by Quality Assurance.

Prof. Dr. J. Pauluhn D.A.B.T.
Board Approved Toxicologist (DGPT)
Study Director

May 04, 2006
Date

1. TABLE OF CONTENTS

1. TABLE OF CONTENTS 4
2. QUALITY ASSURANCE STATEMENT 6
3. SIGNATURES 7
4. SUMMARY 8
5. INTRODUCTION 10
6. RESPONSIBILITIES 11
7. MATERIALS AND METHODS 12
7.1. TEST SUBSTANCE 12
7.2. TEST SYSTEM AND ANIMAL MAINTENANCE 12
7.3. STUDY DESIGN 14
7.4. AEROSOL GENERATION AND EXPOSURE TECHNIQUE 15
7.5. INHALATION CHAMBER TEMPERATURE AND HUMIDITY 17
7.6. ANALYSIS OF THE TEST ATMOSPHERE 18
7.7. CHARACTERIZATION OF AERODYNAMIC PARTICLE-SIZE DISTRIBUTION 18
7.8. COLLECTION EFFICIENCY 20
7.9. STABILITY OF THE TEST ATMOSPHERE 21
7.10. BODY WEIGHTS 21
7.11. CLINICAL SIGNS 22
7.12. DELAYED-ONSET LUNG FUNCTION MEASUREMENTS 22
7.13. BRONCHOALVEOLAR LAVAGE 22
7.14. IMMUNOLOGICAL DETERMINATIONS 23
7.15. ORGAN WEIGHT 23
7.16. NECROPSY 23
7.17. HISTOPATHOLOGY 23
7.18. STATISTICAL EVALUATION 23
7.19. REPRODUCTION OF RAW DATA 24
7.20. SOFTWARE PROGRAMMING AND VALIDATION 24
7.21. RAW DATA AND REPORT ARCHIVAL 25
8. RESULTS 26
8.1. TOPICAL INDUCTION AND INHALATION CHALLENGE 26
Clinical Findings 27
Body weights 28
8.2. ELICITATION OF RESPIRATORY HYPERSENSITIVITY – MDI-CHALLENGE 28
8.3. LUNG AND LYMPH NODE WEIGHTS 35
8.4. BRONCHOALVEOLAR LAVAGE 35
8.5. IGE-DETERMINATIONS IN SERUM 37
8.6. NECROPSY 38
9. DISCUSSION 39
10. KEY TO ABBREVIATIONS IN TABLES 41
11. REFERENCES 42

12. APPENDIX **43**

Scheduling / Activities *43*
Topical Induction - Dose Calculation *44*
Inhalation Challenge – Exposure Details *49*
Inhalation Challenge – Mean Exposure Concentrations *56*
Particle size Distribution *57*
Clinical Observations *71*
Summary of Clinical Findings during Induction / Challenge *147*
Body Weights *149*
Analysis of Breathing Patterns (Repeated Challenge) *155*
Analysis of Breathing Patterns (Terminal Challenge) *159*
Bronchoalveolar Lavage *205*
Lung Lavage / Statistical Analysis *231*
Lung Lavage / Individual Reports *296*
IgE-Determinations *308*
IgE-Determinations – Test Report *317*
LALN Weights *326*
Gross Pathology Findings *335*
Chow Specification - Nutrients *340*
End of Report *341*

Quality Assurance Statement

Test Item : POLYMERIC MDI

Study No.: T4075868

Study-based inspections were conducted by the Quality Assurance on the dates given below. Audit reports have been submitted in writing to the study director and, if necessary, also to the laboratory management, or other persons affected.

Date of Inspection	Study phases inspected	Date of report to study director and/or management
Aug. 08, 2005	STUDY PLAN	Aug. 08, 2005
Aug. 12, 2005	MEASUREMENTS	Aug. 12, 2005
Aug. 18, 2005	ADMINISTRATION, EVALUATION, RAW DATA	Aug. 18, 2005
Sept. 14, 2005	ADMINISTRATION, MEASUREMENTS, RAW DATA	Sept. 15, 2005
Nov. 04, 2005	MEASUREMENTS, RAW DATA	Nov. 04, 2005
Nov. 08, 2005	MEASUREMENTS, RAW DATA	Nov. 08, 2005
Nov. 09, 2005	NECROPSY, SAMPLING OF SPECIMENS, RAW DATA	Nov. 09, 2005

The raw data and the final report were not audited by Quality Assurance.

Quality Assurance Unit
BHC-PS-PH-QA-GLP

Date: May 4, 2006 **Responsible:** Rauxel-Dornik
Dr. M. Rauxel-Dornik

3. SIGNATURES

Study Director - Prof.Dr. J. Pauluhn D.A.B.T.:

..... Date: May 4, 2006

Head of Section - Dr. Dr. H.-J. Ahr:

..... Date: May 4, 2006

4. SUMMARY

Brown Norway rats (eight male per group) were sensitized topically on days 0 and 7 to undiluted polymeric methylenediphenylene diisocyanate (abbreviated MDI). The total dose administered ranged from 25 to 694 mg MDI/kg bw using variable surface areas. For elicitation of respiratory allergy the rats were challenged by inhalation to 38±3 mg MDI-aerosol/m³ (duration 30 min) on target days 21, 35, 49, and 75. The time spacing between each challenge was long enough to minimize acute irritation-related carry-over effects. Two non-sensitized control groups were used in this study; one was repeatedly challenged similar to the groups sensitized topically to MDI and the other was not aerosol challenged at any time point. The assessment of respiratory changes delayed in onset was determined in one single group and demonstrated a clear dependency of frequency of challenge exposures and the magnitude of changes. This analysis shows that a minimum of four challenge exposures is apparently required to elicit unequivocal changes in delayed-onset breathing patterns suggestive of a MDI-induced asthmagenic response. The challenge dose chosen was minimally to slightly irritating, which was supported by the small differences in breathing patterns and BAL-endpoints observed between the non-challenged and re-challenged control groups. In a previous study using essentially a similar design of elicitation, there was histopathological evidence of acute lung irritation. Therefore, the challenge concentration x time relationship chosen appears to be in the range of the acute irritation threshold and is suitable to differentiate between irritant and asthmagenic responses. After the final (fourth) challenge with MDI-aerosol, respiratory function measurements focused on the determination of effects delayed in onset. One day after the final challenge, lung and lung-lymph-node weights were determined and the lungs were lavaged for the analysis of inflammatory endpoints by bronchoalveolar lavage (BAL). Total IgE was analyzed in serum.

The results of study can be summarized as follows: Results from bronchoalveolar lavage analysis and obtained by breathing pattern analysis for approximately 20 hours post-challenge were suggestive of an unequivocal asthmagenic response. This was indicated by changes in BAL and included elevated protein, increased numbers of neutrophilic and eosinophilic granulocytes and total cell counts. Lung weights (absolute and relative) and lung-lymph-node weights were increased. Despite the marked differences in dosages and surface areas utilized to sensitize the animals, throughout the groups sensitized to and challenged 4-times with MDI, the magnitude of responses was essentially similar. Most changes gained statistical significance when compared with the non-sensitized and re-challenged control group. Lung

weights and BAL endpoints of non-sensitized/re-challenged and non-sensitized/not-challenged were not at variance, indicating that the concentration regimen used for challenge did not produced irritant-related confounding effects. Total IgE determinations did not reveal statistical differences amongst the groups. Animals induced topically (694 mg MDI/kg bw) and challenged approximately 3 months later displayed a borderline increase of indicators suggestive of an MDI-mediated asthmagenic response.

In summary, the findings of this study support the conclusion that the Brown Norway rat model is suitable to identify MDI as an asthmagenic agent upon moderate to high topical induction dosages followed by repeated inhalation challenge exposures to mildly irritant concentrations of MDI. Consistent and unequivocally positive delayed-type changes of breathing patterns were observed in sensitized rats. These findings suggest that MDI promotes a more delayed-onset type rather than immediate-type inflammatory response. However, there are limitations with regard to the current dosing procedures used for the sensitization of animals as that the total dose administered is undoubtedly linked to the surface area. This means both variables are interrelated. Accordingly, is appears to be difficult to disentangle unequivocally the role of 'total dose' *versus* 'surface area dose' in this animal model.

5. INTRODUCTION

The objective of this study was to evaluate the asthmagenic potential of MDI using Brown Norway rats sensitized to MDI, administered by topical exposures. For the elicitation of respiratory allergy a more chronic repeated challenge protocol was chosen. The advantage of a repeated challenge protocol is that features characteristic of the allergic airway, that include airway remodeling and sustained recruitment of inflammatory cells, can be suitably evaluated and assessed. However, due to the available data from previous studies, histopathology was omitted.

Testing facility:

The study was conducted at Bayer HealthCare AG, PH-PD Toxicology International / Inhalation Toxicology, D-42096 Wuppertal, Germany.

Study/project identification:

Bayer Project-no.:	T4075868
III- Project-no.	256-EU-MTX
Experimental starting date:	August 05, 2005
Study starting date:	August 09, 2005 (first exposure of animals)
Experimental completion date:	January 03, 2006
Study completion date:	see signature of study director (page 7)

6. RESPONSIBILITIES

Air conditioning/air make-up.....D.I. F.-W. Mentzel
Archiving of raw data and report: .....R. Zils
Analytical characterization of test article:.....Dr. J. Kautz/BMS-Uerdingen
Biometric Evaluation of Data:.....Prof. Dr. Dr. J. Pauluhn
Bronchoalveolar lavage:.....Dr. I.Loof
Cell count & cytospins:.....Dr. H. Ellinger Ziegelbauer
Gross Pathology/Necropsy: .....Prof.Dr. M. Rosenbruch
Head of Institute:.....Dr. v.Keutz
Head of Section: .....Dr.Dr. H-J.Ahr
Immunological Determinations (IgE): .....Prof. Dr. H.W.Vohr
Laboratory Animal Services: .....Dr. W. Feller
Quality Assurance:.....Dr. A. Paessens
Study Monitor:.....Dr. Mike Woolhiser, Dow Chemicals, U.S.A.
Study Director and Report Author:.....Prof. Dr. Dr. J. Pauluhn

7. MATERIALS AND METHODS

7.1. Test Substance

Chemical name:	Diphenylmethane-4,4'-diisocyanate (MDI-polymer)
Abbreviation:	MDI
Commercial name:	MDI 44 V 20 L
Batch-no.:	P4DB000336 (Tox Id: 9520)
Purity:	47.15% Monomic MDI (4,4'-MDI: 42.2%); NCO-content: 31.08%
Date of production:	shelf life: verified up to December 26, 2005.
Manufacturer:	BAYER Material Science AG, Leverkusen, Germany
Storage conditions:	refrigerator (≈ 4 °C) / darkness / N_2-atmosphere {prior to study}
Storage conditions:	at room temperature {during study}.
Handling:	complete exclusion of air/humidity (handling and storage in dry nitrogen)
Appearance:	brownish, translucent liquid material (viscous)

7.2. Test system and animal maintenance

Species: Male Brown Norway rats of the strain BN/Crl BR were purchased from Charles River, Sulzfeld, Germany. At the commencement of the study the mean body weights of all rats were approximately ≈235 g.

Acclimatization: The animals were acclimatized to the animal room conditions for approximately 1 week before.

Identification: Animals were identified by both individual color-marking and cage-labels.

Randomization: Before the start of the study the health status of each animal was assessed. Animals were subsequently assigned to exposure groups at random (randomization procedure *vide infra*).

Health status: Only healthy animals free of signs were used for this study. The animals were not vaccinated or treated with anti-infective agents either before their arrival or during the acclimatization or study periods.

Animal housing: During the acclimatization and study periods the animals were housed singly in conventional Makrolon® Type II cages (based on A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)). Cages were changed twice a week while unconsumed feed and water bottles were changed once per week. The legal requirements for housing experimental animals (Directive 86/609 EEC) were followed.

Bedding: Bedding consisted of type BK 8/15 low-dust wood granulate from Ssniff, Soest/Westfalen, Germany. The wood granulate was randomly checked for harmful constituents at the request of the Laboratory Animal Services, Bayer HealthCare AG.

Animal rooms: All animals were housed in a single room.

Environmental Conditions in the Animal Room

The animal room environment was as follows:

Room temperature:	22 ± 2 °C
Relative humidity:	approximately 50 %
Dark/light cycle:	12 h/12 h; artificial light from 6.00 a.m. to 6.00 p.m. Central European Time
Light intensity:	approximately 14 watt/m² floor area
Ventilation:	approximately 10 air changes per hour

The room humidity and temperature were continuously monitored and documented using a calibrated thermohygrograph. Occasional deviations from these conditions occurred, e.g. as a result of animal room cleaning, but these had no detectable influence on the outcome of this study.

Cleaning, disinfection, and pest control: The animal room was regularly cleaned and disinfected once a week with neat TEGO® 2000. Contamination of the feed and contact with the test system were excluded. Pest control measures using pesticides were not taken in the animal room.

Feeding: Ration consisted of a standard fixed-formula diet (KLIBA 3883 = NAFAG 9441 pellets maintenance diet for rats and mice; PROVIMI KLIBA SA, 4303 Kaiser-augst, Switzerland) and tap water (drinking bottles). Both food and water were available *ad libitum*. The pelletized feed was contained in a rack in the stainless-steel

wire cage cover. The nutritive composition and contaminant content of the standard diet was checked regularly by random sampling by the Laboratory Animal Services, Bayer HealthCare AG. Details concerning general feed specification are provided in the Appendix.

Water: Drinking quality municipality tap-water (current versions of the Drinking Water Decree (TrinkwV)) was provided *ad libitum* in polycarbonate bottles containing approximately 300 ml (based on A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)). The results of feed and water analyses are retained by Bayer HealthCare AG. The available data provided no evidence of an impact on the study objective.

7.3. Study design

The protocols utilized to sensitize Brown Norway rats to MDI were largely consistent with the methods described previously for similar investigations in Brown Norway rats sensitized to trimellitic anhydride (TMA) (Pauluhn et al., 2002) and MDI.

This study consisted of two naïve control groups and ten groups of BN rats that were sensitized epicutaneously on days 0 and 7. Each group consisted of eight male rats. The skin was shaved 1 day prior to administration. The different doses were administered by dosing MDI to aluminum foil spots (for diameter and numbers/animal see Table 1). After metering a predefined volume of MDI to each foil the weight of MDI was determined using a digital balance. The test substance was then transferred to the skin by pressing the spot onto the skins' surface and was then removed. Each foil spot was re-weighed to confirm the actual dose applied (details are shown in the Appendix pp. 56). Rats were prevented from grooming or scratching by wearing an Elizabean collar up to the morning the day following administration (Buster Birdcollars; Kruuse, DK, Cat no.: 273375).

The control group no. 11 was neither sensitized nor challenged at any time point, whilst the control no. 1 was repeatedly challenged with approximately 38 mg MDI-aerosol/m^3 on target days 21, 35, 49, and 75 (exact days are shown in the Appendix) for 30-min. At these time points, four out of the eight rats of the group no 4 rats were monitored for 20 hours one day before and shortly after the MDI challenge for delayed onset respiratory effects in order to assess an progression of changes. Animals of the group nos. 1- 10 were challenged in the same way, however, measurements for delayed-onset responses commenced shortly after the final (4th) challenge with MDI-aerosol. Rats of group 12 received one single challenge only

within the time range groups 1-10 were challenged for the fourth time. One day after the final MDI-challenge, all rats were sacrificed, the weights of exsanguinated lungs and lung lymph nodes were determined and the lungs were lavaged for the analysis of endpoints suggestive of an inflammatory response. Lavaged lungs were preserved in buffered formalin but not examined by histopathology. At sacrifice blood was collected by heart puncture for total IgE determination in serum. The dosing regimen is shown in Table 1.

Table 1: Target dosing regimen and group allocation

Group (8 rats/group)	Spots/Sessions #	Spot-Size cm	Dose/spot ul	Total Dose ul/cm²	Total Dose ul/rat	Total Area cm²/rat	Challenge
1	-	-	0	0.0	0.0	–	1-2-3-4
2	4	2	10	3.2	80.0	25.1	1-2-3-4
3	2	2	20	6.4	80.0	12.6	1-2-3-4
4	4	1	10	12.7	80.0	6.3	1-2-3-4
5	1	2	10	3.2	20.0	6.3	1-2-3-4
6	2	1	5	6.4	20.0	3.1	1-2-3-4
7	1	1	10	12.7	20.0	1.6	1-2-3-4
8	1	1	2.5	3.2	5.0	1.6	1-2-3-4
9	2	0.5	1.25	6.4	5.0	0.8	1-2-3-4
10	1	0.5	2.5	12.7	5.0	0.4	1-2-3-4
11	-	-	0	0.0	0.0	–	0-0-0-0
12	4	2	20	6.40	160	25.1	0-0-0-1

Challenge: 0: no challenge

7.4. Aerosol Generation and Exposure Technique

Mode of exposure: Animals were exposed to the aerosolized test substance in restrainers made of Plexiglas. Restrainer tubes were chosen that accommodated the animal's size. The design of the *directed-flow* inhalation chamber prevents re-breathing of the test atmosphere (Moss and Asgharian, 1994). This type of exposure is preferable to whole-body exposure on scientific (Pauluhn, 1984) and technical reasons (rapid attainment of steady-state concentrations, no problems with regard to test atmosphere inhomogeneities, better capabilities to control all inhalation chamber parameters, easier cleaning of exhaust air, and lower consumption of test item). Moreover, contamination of the hair-coat can largely be avoided. The operation of this commercially available chamber (TSE company in Bad Homburg v.d.H., Germany) and its validation has been published in detail (Pauluhn, 1994).

Generation of atmosphere: Atmospheres of MDI for inhalation exposures were generated under dynamic conditions using a digitally controlled Hamilton Microlab M

pump and a modified Schlick-nozzle Type 970, form-S 3 (Schlick GmbH, Coburg, Germany).

Generation of aerosol: The test substance was nebulized using conditioned (dry, oil-free) compressed air (dispersion pressure approximately 600 kPa, 10 µl MDI/min, 15 L/min and inhalation chamber segment). The nozzle were maintained at approximately 40 °C by a water jacket connected to a digitally controlled JULABO thermostat. The increase of temperature within the nozzle resulted in a marked decrease in viscosity and hence increased reproducibly the output of aerosol. The respective concentration was achieved by applying the extraction/dilution cascades depicted in Fig. 1.

Inhalation Chamber: Each segment of the aluminum inhalation chamber has the following dimensions: inner diameter = 14 cm, outer diameter = 35 cm (two-chamber system), height = 25 cm (internal volume = about 3.8 L). The construction of the inhalation chamber is shown schematically in Fig. 1. For this study a two segment-chamber was used. Flow rates through the inhalation chamber were 30 L/min. Further details are presented in the Appendix.

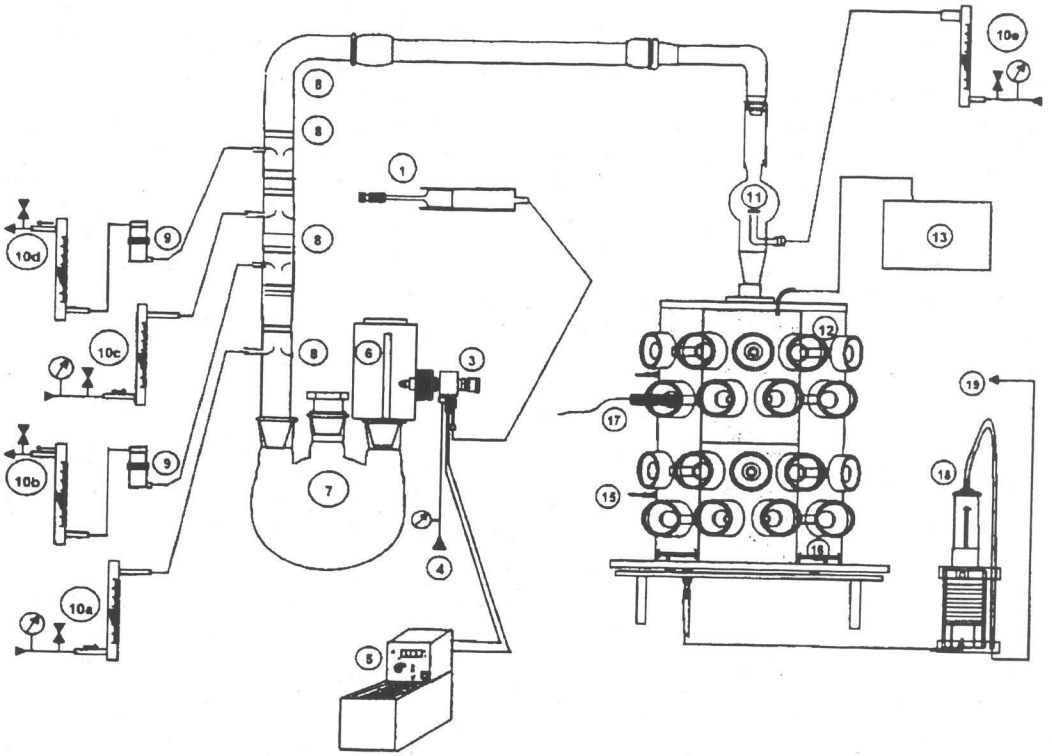
Compressed air conditioning: The compressed air was produced with two Boge Model SB 270/15/350D compressors operated in parallel. The air was automatically conditioned (i.e. water, dust and oil removed) by subsequent passage through a VIA compressed air dryer. The regulated operating pressure of the compressors was 8 - 10 bars (800 - 1000 kPa). Pressure-reduction valves were used to set the operating pressure.

Inhalation chamber - steady-state concentration: The test atmosphere generation conditions assured at least 230 air volume exchanges per hour. A steady state was established in less than approximately one minute of operation under these test conditions ($t_{95\%}$ = 3 x chamber volume/air flow rate; McFarland, 1976). The ratio of input to exhaust air was selected to ensure that approximately 90% of the input air was removed by the exhaust system, and the remainder via other chamber openings. An air flow towards the rats' exposure zones was thus provided in the exposure system (*directed-flow* principle) allowing an adequate ventilation of the animals' breathing zone.

Air flows: During the exposure period air flows were monitored continuously and, if necessary, readjusted to the conditions required. Air flows were measured with calibrated flow-meters and/or soap bubble meter (Gilibrator, Ströhlein Instruments, Kaarst) and were checked for correct performance at regular intervals.

Treatment of exhaust air: The exhaust air was purified via cotton-wool/HEPA filters. These filters were disposed of by Bayer AG.

Figure 1: Inhalation Chamber (schematic)



1. MDI-reservoir and Harvard PHD 2000 Pump	11. Mixing unit (glass reservoir)
3. Schlick-nozzle (@ 40°C)	12. Directed-flow nose-only exposure zone
4. Pressurized, dry, conditioned air with pressure gauge	13. Photometer (real-time aerosol monitoring)
5. JULABO thermostat– water bath (water jacket)	15. Sampling for nitro-reagent/filter analyses
6. PVC – pre-separator	16. Inhalation chamber exhaust location
7. Mixing unit (3-neck glass flask)	17. Temperature-/humidity sensor
8. Dilution cascade	18. Cotton-wool aerosol filter + HEPA filter
9. Cotton-wool aerosol filter	19. Exhaust air
10.a-e. Dilution air flows	

7.5. Inhalation Chamber Temperature and Humidity

Temperature and humidity measurements were made using a computerized system (Hydra, Fluke-Philips). The values were recorded at intervals of 5 min (computerized recording). The test atmosphere temperature and humidity were measured at the

exposure location (see Fig. 1) using a FTF-sensor (Elka-Elektronik, Lüdenscheid). The sensor was calibrated using saturated salt solutions according to Greenspan (1977) and Pauluhn (1994) in a two-point calibration at 33% ($MgCl_2$) and at 75% (NaCl) relative humidity. The calibration of the temperature sensor is also checked at two temperatures using reference thermometer. The measured values were evaluated using spreadsheet software.

7.6. Analysis of the Test Atmosphere

Nominal concentration: The nominal concentration was calculated from the ratio of the quantity of test item atomized. Specific information concerning air flows and test atmosphere concentrations are provided in the Appendix.

Gravimetric concentration: The test-item concentration was determined by gravimetric analysis (filter: Glass-Fibre-Filter, Sartorius, Göttingen, Germany; digital balance). The total volume sampled per analysis was 80 L (sampling flow rate 4 L/min).

Chamber samples were taken in the vicinity of the breathing zone (see Fig. 1). The number of samples taken was sufficient to characterize the test atmosphere and was adjusted so as to accommodate the sampling duration and/or the need to confirm specific concentration values. Optimally, samples were collected after the equilibrium concentration had been attained in hourly intervals. All analytical concentrations reported refer to mg MDI/m^3 air.

7.7. Characterization of Aerodynamic Particle-Size Distribution

The samples for the analysis of the particle-size distribution were also taken in the vicinity of the breathing zone. The results of the individual determinations are shown in the Appendix.

The particle-size distribution was analyzed using a BERNER-TYPE AERAS low-pressure critical orifice cascade impactor (Hauke, Gmunden, Austria). Specifications and evaluations are provided in the Appendix. The individual impactor stages had been covered by an aluminum foil which was subjected to gravimetric analysis An adhesive stage coating (silicone spray) was not used to prevent particle bounce and re-entrainment because of the physical properties of the test compound. Gravimetric analyses were made using a digital balance.

The parameters characterizing the particle-size distribution were calculated according to the following procedure:

Mass Median Aerodynamic Diameter (MMAD): Construct a 'Cumulative Percent Found - Less Than Stated Particle Size' table, calculate the total mass of test item collected in the cascade impactor. Start with the test item collected on the stage that captures the smallest particle-size fraction, and divide this mass of the test item by the total mass found above. Multiply this quotient by 100 to convert to percent. Enter this percent opposite the effective cut-off diameter of the stage above it in the impactor stack. Repeat this step for each of the remaining stages in ascending order. For each stage, add the percentage of mass found to the percentage of mass of the stages below it. Plot the percentage of mass less than the stated size versus particle size in a probability scale against a log particle-size scale, and draw a straight line best fitting the plotted points. A weighted least square regression analysis may be used to achieve the best fit. Note the particle size at which the line crosses the 50% mark. This is the estimated Mass Median Aerodynamic Diameter (MMAD).

Calculation of **Geometric Standard Deviation (GSD)**: Refer to the log probability graph used to calculate the Mass Median Aerodynamic Diameter. Provided that the line is a good fit to the data, the size distribution is log normal, and the calculation of the Geometric Standard Deviation is appropriate. Note that particle size at which the line crosses the 84.1% mark. Note the particle size at which the line crosses the 50% mark and calculate as follows: GSD = 84.1% mark / 50% mark.

To verify graphically that the aerosol is in fact unimodal and log-normally distributed the normalized mass per stage (f_H') is evaluated as a histogram. $\Delta \log D_p$ is equal the difference $\log D_{p+1} - \log D_p$, whereas D_p is the lower cut-size limit and D_{p+1} the higher cut-size limit of the corresponding impactor stage. Calculate the histogram f_H' by equation:

$$f_H' = \frac{1}{N_f} \times \frac{mass\ /\ stage}{\Delta \log D_p} \qquad (1)$$

Calculate the log-normal mass distribution $y'(D_{ae}) = 1/N_f \times y(D_{ae})$ as a function of the aerodynamic diameter (D_{ae}) using by equation:

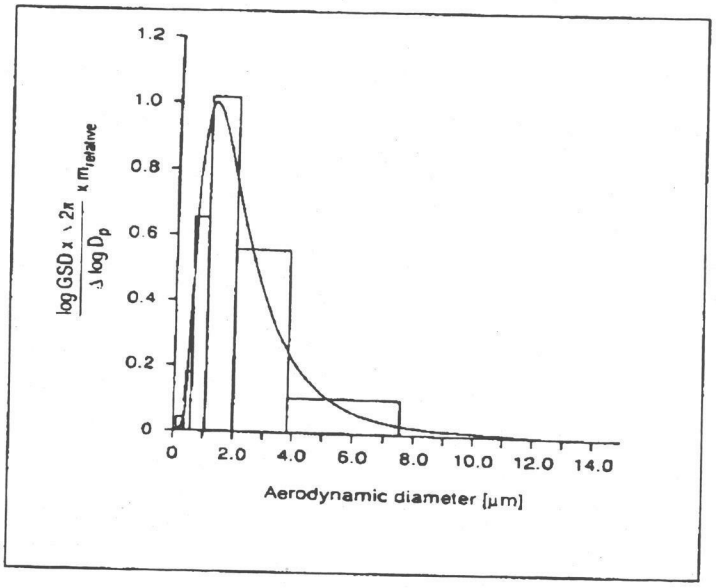
$$y'(D_{ae}) = \exp\left[-\frac{(\log D_{ae} - \log MMAD)^2}{2 \times \log^2 GSD}\right] \qquad (2)$$

and use the normalization factor (N_f):

$$N_f = \left(\frac{\Sigma mass}{\log GSD \times \sqrt{2\pi}} \right)^{-1} \qquad (3)$$

It should be noted that for the graphical display of data the size distributions shown in Fig. 2 is constructed utilizing equation 2.

Figure 2: Principle of characterization of aerosol atmosphere



The relative mass with an aerodynamic diameter ≤ 3 µm ("respirable mass fraction") [Raabe, 1982; Snipes, 1989; SOT-Commentary, 1992] is calculated from the regression line. For probit transformation and linear regression FORTRAN algorithms published by Rosiello *et al.* (1977) are used. The MMAD was calculated using published following formulas (Marple and Rubow, 1980; Pauluhn, 1994; USP XXII, 1992).

The algorithm for the calculation of particle size characteristics is taken from pertinent reference works on aerosol physics (Dennis, 1976; Marple and Rubow, 1980) and proves to be generally applicable (Pauluhn 1988; Pauluhn, 1994).

7.8. Collection Efficiency

The sampling equipment was adjusted with calibrated flow-meters to internationally recognized standards (ACGIH, 1978; Section I "Calibration of Air Sampling Instruments").

The conditions for generating the test atmosphere are optimized to provide maximum aerosol respirability to rats (Raabe, 1982; Snipes, 1989; SOT-Commentary, 1992). The absence of larger particles and high flow rates in the vicinity of the sampling

ports make it possible to disregard potential anisokinetic sampling errors, thus ensuring a representative sampling even with different sampling probe orifice diameters and flow rates. The tolerance limits for the radius of the probe orifice are calculated using the following formula [ACGIH, 1978]. Calculations consider both a particle size distribution that encompasses aerodynamic diameters (D_{ae}) of 0.5 to 7.4 µm and sample flows ranging from 8 to 80 ml/sec.

$$5 \times \sqrt[3]{\frac{flow \times \tau}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times \sqrt[2]{\frac{flow}{g \times \tau \times \pi}}$$

r_p = radius of the sample probe in cm = ½ x D_p
τ = relaxation time (D_{ae} 0.5 µm = 1×10^{-6} sec; D_{ae} 7.4 µm = 1.7×10^{-4} sec)
g = gravity constant = 980 cm/sec^2

Tolerance limits calculations for the sample probe orifice (r_p) indicated that a representative sampling is assured when the orifice inner diameter is in the range of 1.0 to 1.6 cm. Orifices of the sampling instruments used here are in compliance with this criteria. Details of the D_p tolerance limit calculations are published elsewhere (Pauluhn, 1988; Pauluhn, 1994).

7.9. Stability of the Test Atmosphere

The integrity and stability of the aerosol generation and exposure system was measured by using a RAM-1 real-time aerosol photometer (MIE, Bedford, Massachusetts, USA). Samples were taken continuously from the vicinity of the breathing zone.

This chamber monitoring allows for an overall survey of toxicologically relevant technical parameters (inlet and exhaust flows as well as atmosphere homogeneity, temporal stability, and generation performance). Interruptions in exposure (e.g. resulting from obstruction of nozzles or other technical mishaps) are recorded and, if applicable, a commensurate interval is added to the exposure duration for compensation.

7.10. Body weights

The body weights were determined prior to induction, on study days three and seven, and weekly thereafter. Animals were also weighed before necropsy.

7.11. Clinical signs

If applicable, the appearance and behavior of each rat was examined carefully before and after exposure/administration and at least once daily thereafter (including weekends). As can be seen from the Appendix (Scheduling/Activities) on some days no observations were made due to public holidays. Assessments from restraining tubes were made only if unequivocal signs occurred (e.g. spasms, abnormal movements, severe respiratory signs). Following exposure, observations are made and recorded systematically; individual records are maintained for each animal. Cage-side observations included, but were not limited to, changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, somnolence and prostration.

7.12. Delayed-onset Lung Function Measurements

Measurements were conducted with spontaneously breathing, conscious, unrestrained and spontaneously breathing rats through a barometric whole-body plethysmography system (Buxco, Troy, NY, USA). Measurements commenced shortly after the MDI-challenge. Briefly, each rat was placed in a chamber, and continuous measurement of the box pressure–time wave was made via a transducer connected to a computer data-acquisition system. Measurements focused on changes in RR (respiratory rate), TV' (pseudo-volume), and Penh (enhanced pause).

7.13. Bronchoalveolar lavage

Shortly after exsanguination, the diaphragm was incised and the lungs were allowed to collapse. The excised lungs of the animals were then lavaged twice with 5 ml saline (kept @ 37 °C) per rat and the 2 washings combined. In the bronchoalveolar lavage fluid (BALF) the following indicators of pulmonary effects were addressed: (1) total protein to quantitate increased permeability of the alveolar-capillary barrier, (2) lactate dehydrogenase (LDH) as an index of cytotoxicity, (3) the total number of cells, and (4) cytodifferentiation with particular focus on eosinophilic and neutrophilic granulocytes. For determination, the cellular content of the lavage fluid was removed by centrifugation at 200 g (10-min @ 4 °C), and the cell pellet was re-suspended in Dulbecco's calcium and magnesium containing phosphate buffered saline (PBS)

substituted with bovine serum albumin (BSA). The number of cells in BAL, including their corpuscular volume, were determined in triplicates on re-suspended cells (Schärfe-System, Casy 1, Reutlingen, Germany).

7.14. Immunological Determinations

Total IgE in serum was determined as detailed in the Appendix (IgE: pp. 308).

7.15. Organ Weight

Following exsanguination (see 7.16 Necropsy), the weights of lungs and lung-associated lymph nodes were recorded.

7.16. Necropsy

Intraperitoneal injection of sodium pentobarbital (Narcoren®) was used for euthanasia. The animals were then examined for gross pathologic changes. All findings deviating from normal were documented. The lungs of the exsanguinated animals were weighed. Complete exsanguination was performed by severing the *aorta abdominalis*. Further details concerning the histopathological evaluation are provided in the respective Appendix.

7.17. Histopathology

For future histological examination to be decided by the sponsor), the lungs were fixed in neutral, buffered formaldehyde.

7.18. Statistical Evaluation

Relative and absolute organ weights, and lavage data were analyzed by a one-way analysis of variance and Tukey-Kramer *post hoc* test (BCTIC Computer Code Collection - Biomedical Computing Technology Information Center: ANOVA a

FORTRAN Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville, Tennessee, USA).

One-way analysis of variances (ANOVA): In this parametric method, the data are checked for normal distribution by comparison of the median and mean values. The variances between the groups were tested for homogeneity with Box's test. If the F-test showed that the variation within the group was greater than that between the groups, this fact is indicated in the appendix by the remark "no statistical difference between the groups". If a difference was determined, a pairwise *post-hoc* (one and two-tailed) comparison of the groups was performed using the Games and Howell modification of the *Tukey-Kramer* significance test.

Randomization: The randomization lists were produced with the aid of a computer program which used a random number generator.

7.19. Reproduction of Raw Data

Raw data entered into, processed by and/or stored in a computer system could be saved and printed out in various formats. The precision (number of decimal places) of the values printed and reproduced in this report reflect toxicologically relevant levels of precision. Deviations between manually calculated and computer-determined values can arise due to rounding. Values with no decimal places do not necessarily represent the pertinent measurement precision of the detection system.

7.20. Software Programming and Validation

Software code for the following purposes was written in Digital Fortran: particle-size analysis, ANOVA, Fisher test, meta-analysis of pulmonary function data. The computer programs were carefully validated. The validation was conducted using text book data sets (Gad and Weil, 1982). However, it should be taken into account that the formal requirements of the GLP-principles for validation of computer software are not fulfilled. Wherever possible, raw data and calculated values are displayed graphically to provide a versatile opportunity for data comparison.

7.21. Raw Data and Report Archival

The study protocol, raw data, and the final report are retained in the archives specified by Bayer HealthCare, Bayer AG. The storage of a retention sample of the test item and, if applicable, also of the reference item is in the responsibility of the sponsor.

8. RESULTS

8.1. Topical Induction and Inhalation Challenge

Twelve groups of eight male rats each were used in this study (see Table 2). Animals of the control groups were not sensitized (group 1 and re-challenged in the same manner as the animals of groups 2-10; group 11: normal housing no challenge exposures. Topical administrations were made as follows - day 0 and 7 using undiluted MDI on the contralateral dorsal area of the trunk (group 2-10, 12). Starting with day 20, rats of all groups (except group 11 and 12) were challenged by inhalation to a mean concentration of 37.9±3 mg MDI/m³ for a duration of 30-min on the target days 21, 35, 49, and 75. Group 12 was received one single challenge only on approximately day 75 (the exact challenge schedule is shown in the Appendix (pp. 49). Concentration and particle-size measurements made during or close to the challenge exposures were reproducible throughout this study. Accordingly, this data demonstrate that all challenges were made under essentially identical conditions.

Specific information addressing the analytical (gravimetric filter analyses) monitoring of the aerosol test atmospheres from the breathing zone is provided in the Appendix (pp. 49). The particle size generated was highly respirable (MMAD 1.4-1.6 µm, GSD 1.8-2). The temperature in the inhalation chamber was in the range suggested by the testing guidelines. Humidity values were lower; this was related to the use of dry conditioned air for aerosol dispersion.

Table 2: Induction of animals – Dosing Regimen

Group	Total Dose (mg/kg bw)	Total Dose/Surface Area (mg/cm²)	Dosed Surface Area (cm²/rat)
1	--	--	--
2	403	7.3	25.2
3	342	13.2	12.56
4	375	28.5	6.28
5	95	7.1	6.28
6	99	14.9	1.58
7	97	28.8	1.58
8	26.0	7.7	3.14
9	25.6	7.5	1.58
10	24.9	14.5	0.78
11	--	--	--
12	693.8	13.6	25.2

Clinical Findings

The incidence, intensity, and time course of MDI-related clinical findings, including skin lesions at the application site, are detailed in the Appendix (pp. 71).

Group 1: No findings.

Group 2: Application site: reddened, application site: red encrustations, application site: edema, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns.

Group 3: Application site: reddened, application site: red encrustations, application site: edema, application site: swollen, application site: induration, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns, bradypnea.

Group 4: Application site: reddened, application site: red encrustations, application site: edema, application site: induration, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns, bradypnea.

Group 5: Application site: reddened, application site: red encrustations, application site: edema, application site: swollen, application site: induration, nasal discharge (serous), stridor, breathing sounds, bradypnea.

Group 6: Application site: reddened, application site: red encrustations, application site: induration, nasal discharge (serous), stridor, breathing sounds.

Group 7: Application site: reddened, application site: red encrustations, application site: edema, application site: induration, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns, bradypnea.

Group 8: Application site: reddened, application site: red encrustations, application site: edema, application site: swollen, application site: induration, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns.

Group 9: Application site: reddened, application site: red encrustations, application site: swollen, application site: induration, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns, bradypnea.

Group 10: Application site: reddened, application site: red encrustations, application site: swollen, application site: induration, nasal discharge (serous), stridor, breathing sounds, labored breathing patterns.

Group 11: No findings.

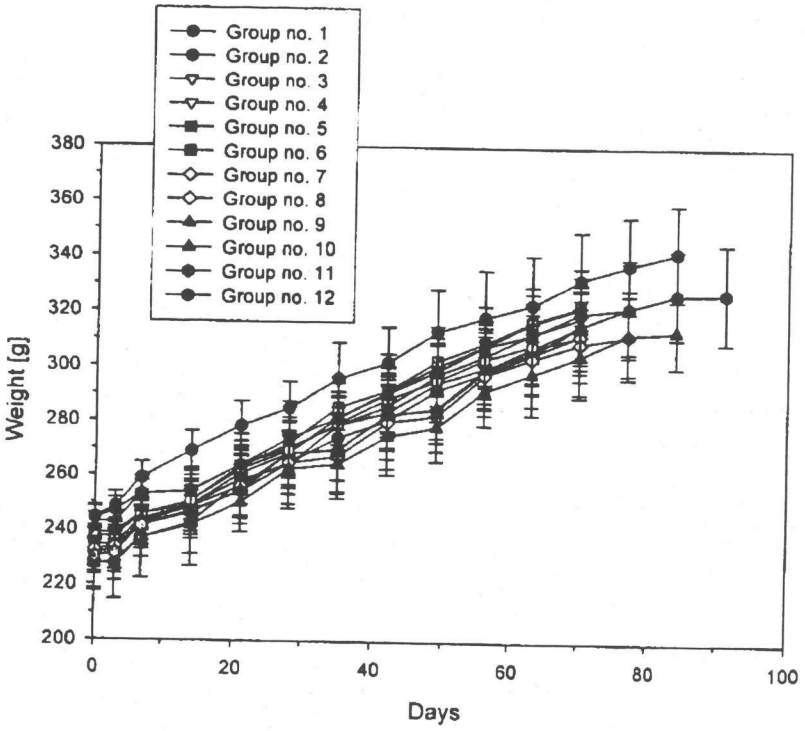
Group 12: Application site: reddened, application site: red encrustations, application site: edema, nasal discharge (serous).

Body weights

Individual data and the mean values (± SD) of the body weights are included in the Appendix (pp. 149). Mean values (± SD) are summarized in Fig. 3.

The data shown in Fig. 3 show that the starting body weight was similar throughout the groups. During the repeated challenge period the body weights of the groups were essentially indistinguishable.

Figure 3: Body weights (means±SD)



8.2. Elicitation of Respiratory Hypersensitivity – MDI-Challenge

Following challenge to MDI-aerosol, four animals per group were simultaneously measured for the occurrence of delayed-onset responses after the final challenge. In group 4 these measurements were performed both before and after challenge (for all challenge exposures).

Delayed onset responses, with focus on area under the curve and individual animal responses are shown in Fig. 4 and 5, respectively. During or following repeated

challenge to approximately 38 mg MDI/m³, the rats sensiti
challenged by repeated inhalation exposures displayed a consist
respiratory response which was more marked with increased nun
as shown . Therefore, the data shown in Fig. 4 (AUC of the Penh e
over the entire measurement period of 20 hours) need t
conservatively. In rats sensitized that were challenged 4-times witl
typical delayed-onset respiratory patterns were distinctly diffe
observed in the non-sensitized rats (group 1). The most salient fir
groups was the characteristic delayed type response peaking appr
after challenge (Fig. 5). During some measurements technical r
during the 20-hour data collection period, e.g., due to uncontrolled
water bottles or the destruction of sealing material due to the gna
rats. The respective data were omitted from evaluation.

Figure 4: Area under the curve (AUC) based on changes of Penh (
before and after challenge (see Table 1 for group allocati

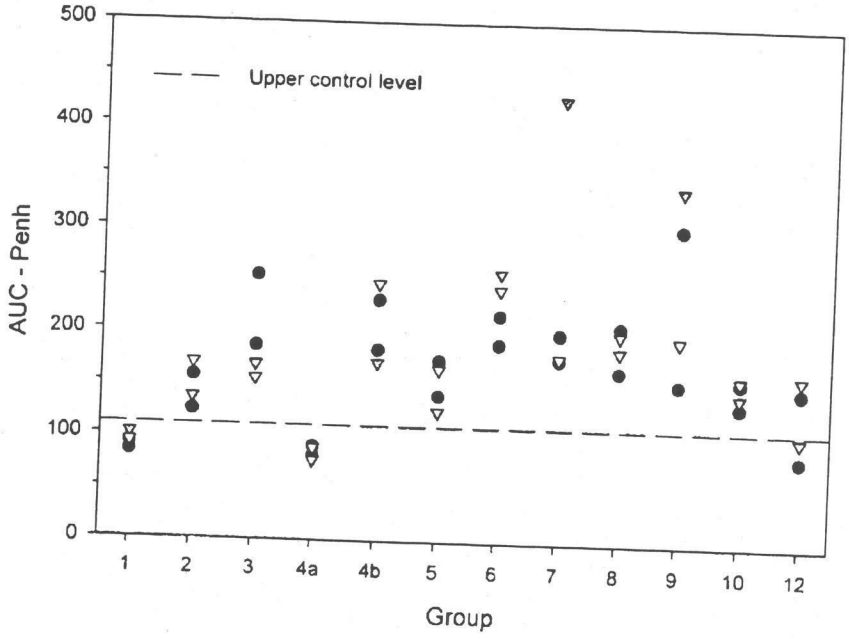


Figure 5: Time course of changes of Penh (enhanced pause) before (group 4 only) and after (all groups) the 4th challenge (see Table 1 for group allocation).

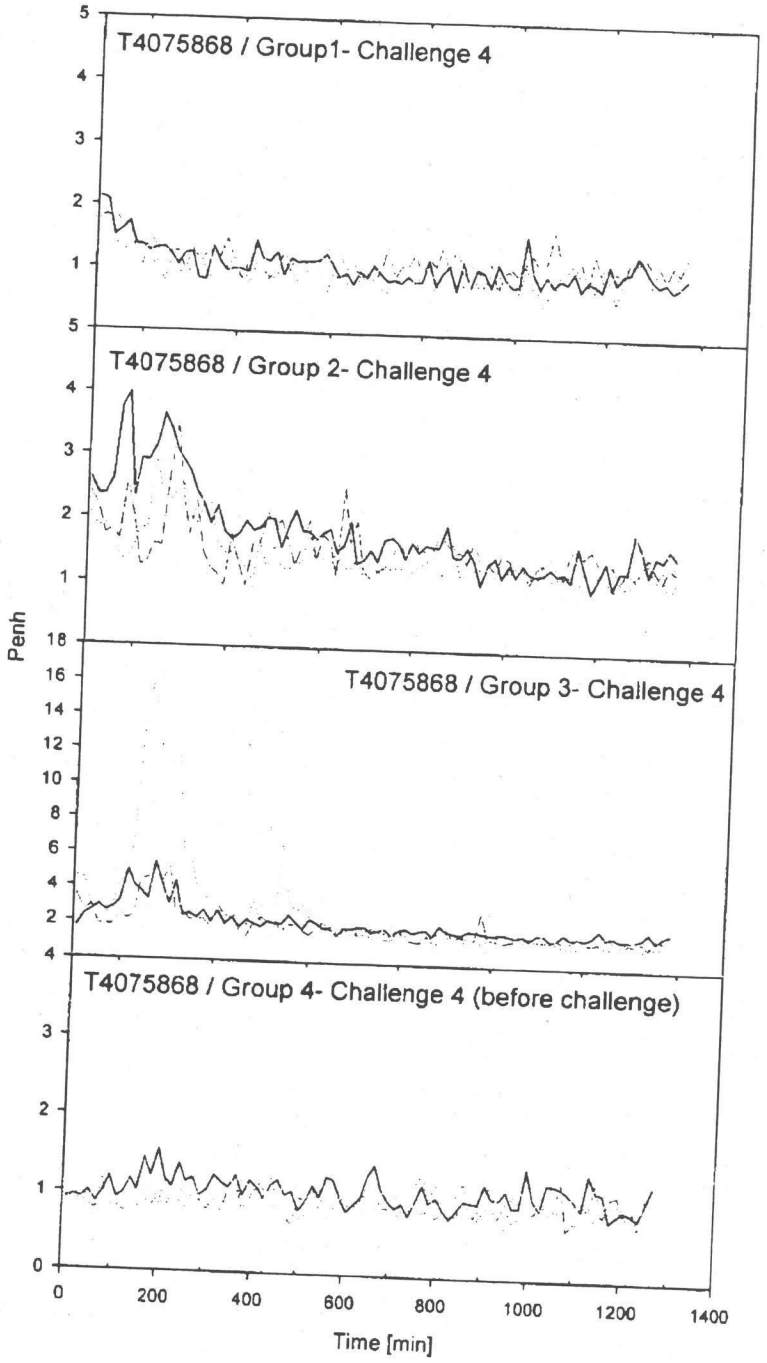


Figure 5: Time course of changes of Penh (enhanced pause) before (group **4** only) and after (all groups) the 4th challenge (see Table 1 for group allocation) - continuation.

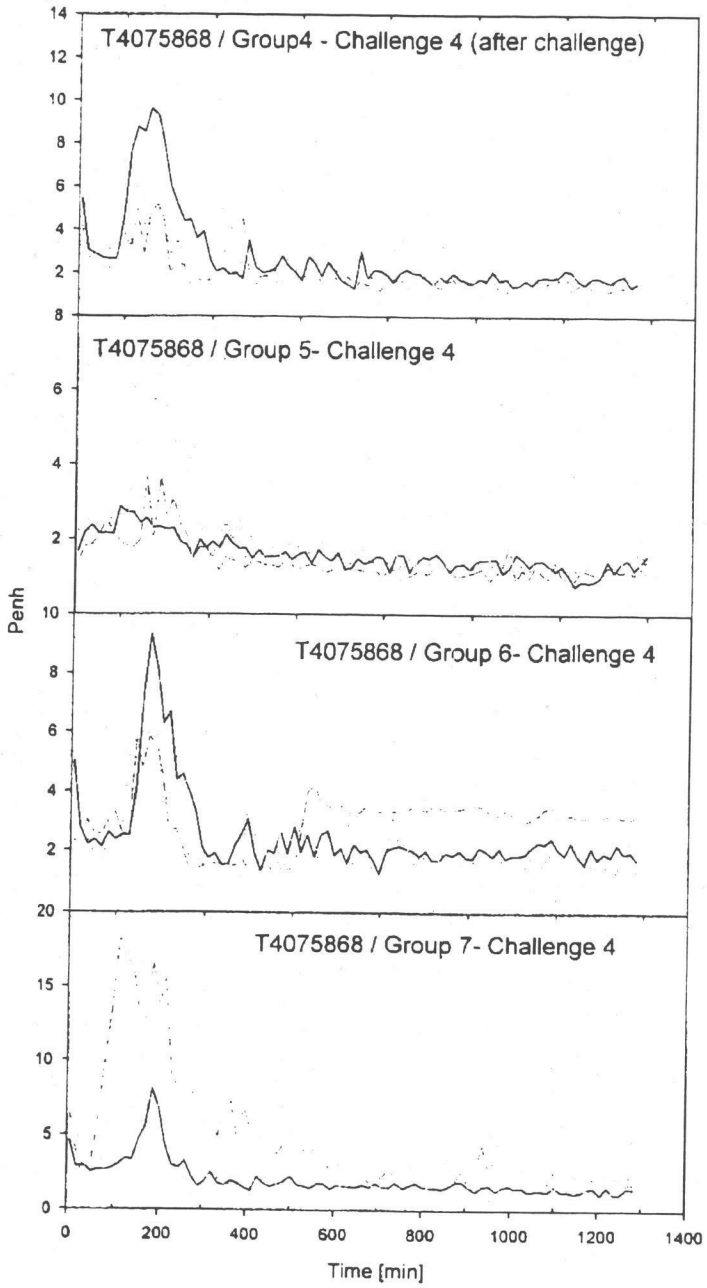


Figure 5: Time course of changes of Penh (enhanced pause) before (group 4 only) and after (all groups) the 4th challenge (see Table 1 for group allocation) - continuation.

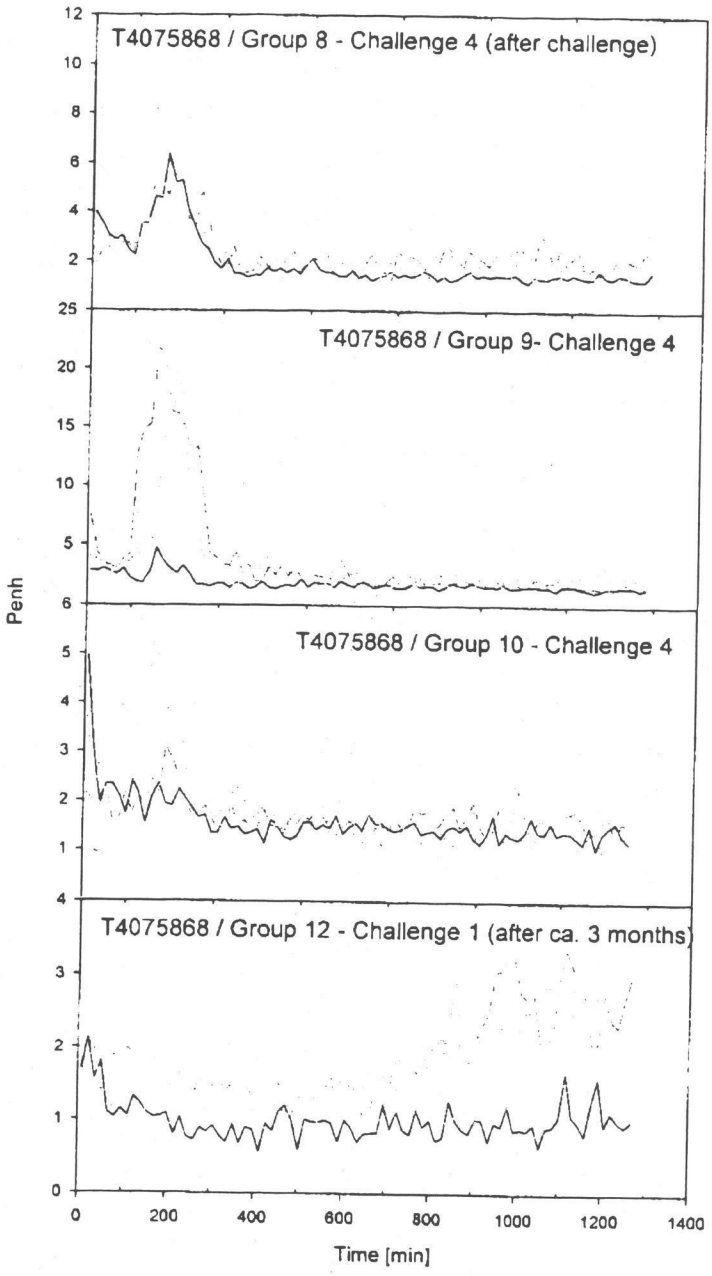


Figure 6: Changes of Penh (enhanced pause) before and after challenge (group 4 only, see Table 1 for group allocation)

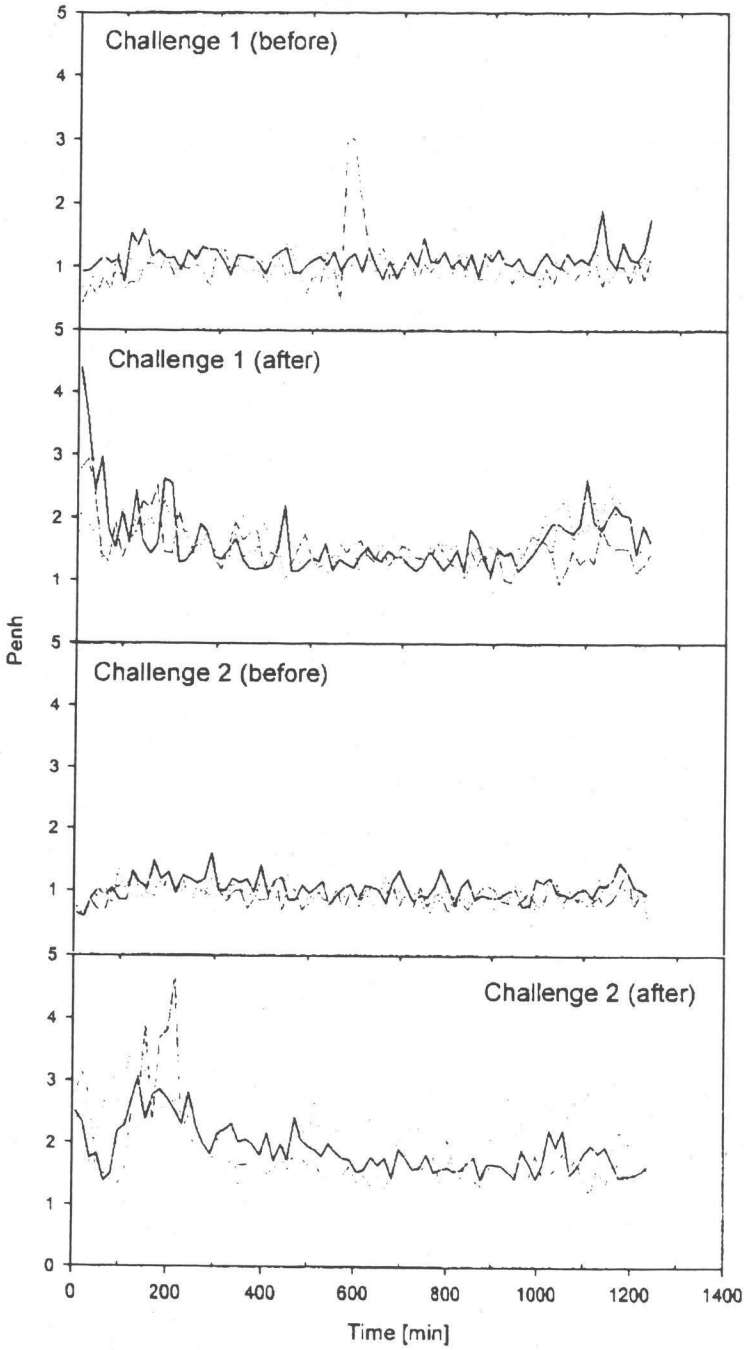
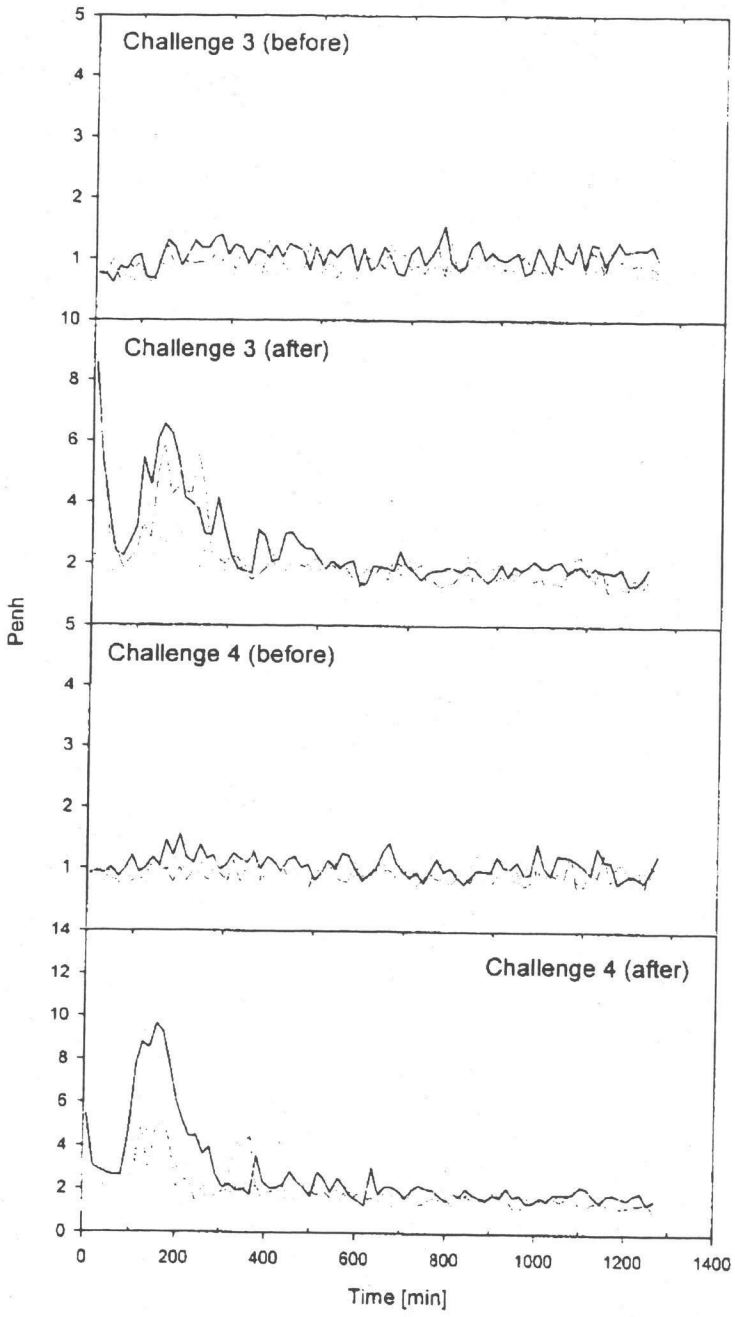


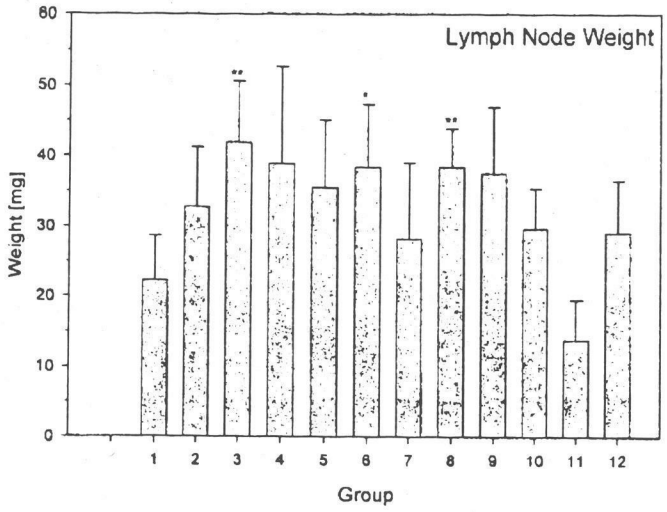
Figure 6: Changes of Penh (enhanced pause) before and after challenge (group 4 only, see Table 1 for group allocation) – continuation.



8.3. Lung and Lymph Node Weights

The absolute and relative lung weights in group 2 – 10 were statistically significant increased (Table 3). In these groups, the lung lymph node weights were somewhat increased (Fig. 7) and some elevations gained statistical significance to the control (group 1). Individual data are shown in the Appendix (pp. 205).

Figure 7: Lymph Node Weights



8.4. Bronchoalveolar Lavage

The results of the bronchoalveolar lavage analysis are detailed in Table 3.

Recovery of bronchoalveolar lavage fluid (BALF) was approximately 80-90% of the instilled volume and was similar amongst the groups. The results summarized in Table 3 show statistically significant changes of most parameters analyzed in groups 2-10. The most prominent changes included BAL-protein, BAL-TCC, BAL-PMN and BAL-eosinophils (for abbreviations see legend of Table 3).

For most endpoints, the challenged control group no. 11 was either not appreciably different or only mildly different from the re-challenged control group no. 1, supporting that the concentration used for challenge did not cause acute irritantion to any appreciable extent. Conclusive dose-dependent related changes were not observed. Rats of group 12 (one singe challenge only) were different from groups 1 and 11, suggesting that a minimal allergic response occurred.

Table 3: Bronchoalveolar Lavage –Mean data

Group	1	2	3	4	5	6	7	8	9	10	11	12
B.W.	345.13	312.13	311.88*	312.38*	321.63	320.25	314.00*	312.13*	317.50	313.50*	314.50*	327.63
LW-abs	1522.88	1816.75**	1924.63**	1921.13**	1826.25**	1910.50**	1969.13**	1884.25**	2021.25**	1872.75**	1386.50	1806.25
LW-rel	442.17	583.75**	618.20**	615.03**	568.07**	596.67**	627.37**	603.66**	637.74**	597.07**	441.25	550.29*
Recov.	8.69	8.38	8.81	8.94	8.63	8.63	9.00	8.88	8.75	8.75	8.75	8.56
TCC	7.27	15.25	13.80	13.47	15.87*	16.21*	16.51*	12.20	11.09	11.84	7.62	7.04
MCD	12.73	11.34*	11.09*	11.09*	11.38*	11.43	11.23*	10.98**	11.06*	11.48	11.89	12.07
MCV	1.48	1.01	0.96	0.92	0.98	1.06	0.99	0.90	0.96	1.13	1.05	1.19
LDH	108.83	145.58	117.51	130.26	142.97	175.24	116.25	176.16	182.03	189.32	58.24	94.77
PROT	0.45	0.77	0.76	0.69	0.72	0.74	0.73	0.79*	0.92*	0.79*	0.27	0.38
Relative:												
AM	92.75	69.79++	65.54++	66.25++	72.50+	65.71++	67.83++	61.13++	55.71++	64.00++	98.21	77.00
PMN	2.96	22.04++	26.46++	26.62++	21.08++	26.38++	25.38++	29.58++	34.92++	26.08++	0.17	15.38++
LYM	0.50	2.54++	4.33++	3.58++	3.00++	2.92++	1.88	3.25++	2.96+	3.00++	0.50	1.46+
EOS	0.29	1.17	1.33	1.29	1.63	1.96	1.75+	2.25++	3.29	3.21++	0.29	1.33
Foamy	0.63	3.17++	1.33	1.21	1.08	2.00++	2.29++	2.96	2.46+	2.58++	0.50	4.54++
NC	2.88	1.29	1.00	1.04	0.71	1.04	0.88	0.83	0.67	1.13	0.33	0.29
Absolute:												
AM	643.68	1048.55	921.26	885.73	1157.44+	1064.63	1119.84+	710.68	592.04	759.69	748.99	534.47
PMN	29.10	348.95++	351.96++	364.96++	326.01++	426.16++	417.29++	392.12++	421.01++	306.57++	1.30++	116.44+
LYM	4.39	39.48++	55.24++	49.14++	48.38++	48.24++	31.11++	40.96++	27.55++	35.60++	3.73	9.40
EOS	2.69	18.13	19.07	17.09	25.95++	31.64++	29.10+	30.03++	29.79++	38.48++	1.81	9.46
Foamy	4.45	45.31++	16.73++	16.74	17.67	33.11++	39.10++	37.37+	29.05+	30.61++	3.89	32.52++
NC	42.65	24.20	15.88	13.77	11.77	17.17	14.84	8.44	9.39	13.45	2.17	2.08

Legend: see next page

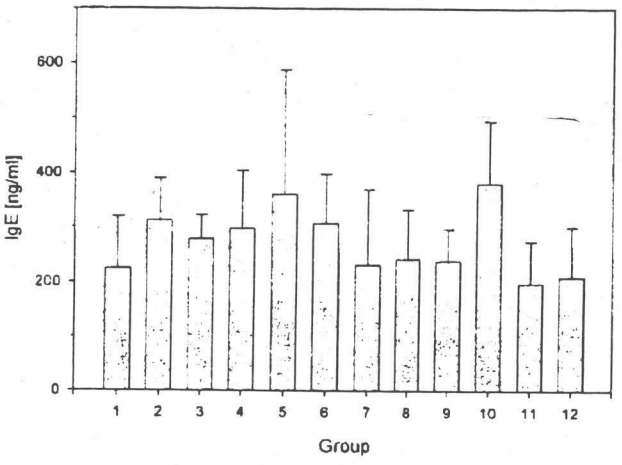
Legend:

```
 B.W.   = Body Weight (bw) at sacrifice - g
LW-abs  = Lung weight (absolute) - mg
LW-rel  = Lung weight (relative) - mg/100 g bw
Recov.  = Recovery of lavage fluid - ml
  TCC   = Total cell count in BAL - # 10^6/lung
  MCD   = Mean cellular diameter - um
  MCV   = Mean cellular volume - 10^-12 L
  LDH   = Lactate dehydrogenase - U/L
 PROT   = Protein - g/L
Count   = Number of cells counted per cytospot
 AM     = Alveolar macrophages - %
 PMN    = Polymorphonuclear cells - %
 LYM    = Lymphocytes - %
 EOS    = Eosinophils - %
Foamy   = Foamy - %
  NC    = Cells not classifiable - %
 AM     = Alveolar macrophages - #10^4/lung
 PMN    = Polymorphonuclear cells - #10^4/lung
 LYM    = Lymphocytes - #10^4/lung
 EOS    = Eosinophils - #10^4/lung
Foamy   = Foamy - #10^4/lung
  NC    = Cells not classifiable - #10^4/lung
*,**    = P < 0.05, P < 0.01 (ANOVA); comparison against group 1
          (values not transformed)
+,++    = P < 0.05, P < 0.01 (ANOVA); comparison against group 1
          (values log-transformed)
```

8.5. IgE-Determinations in Serum

The detailed results, including the respective methodological descriptions are presented in the Appendix (pp. 308). The analysis of data did not reveal any statistically significant difference amongst the groups (Fig. 8).

Figure 8: Total IgE – Means ± SD



8.6. Necropsy

During necropsy, macroscopical lung findings, e.g. area/s, were seen in the majority of animals. A list of the individual findings is included in the Appendix (pp. 335).

9. DISCUSSION

The results of this study showed that two topical induction exposures covering a range of 25-694 mg MDI/kg bw and variable surface area doses resulted in positive responses especially upon the fourth inhalation challenge with approximately 38 mg MDI-aerosol/m³ (challenge duration 30 min). The assessment of respiratory changes delayed in onset in one group demonstrated a clear dependency of frequency of challenge exposures and the magnitude of changes. The challenge dose chosen was considered to be minimally to slightly irritant. This is substantiated by the small, if any, differences in breathing patterns and BAL-endpoints observed in the non-challenged and re-challenged control groups. Previous studies in naïve BN-rats have shown a acute NOAEL for BAL-protein at 900 mg MDI/m³ x min (Pauluhn, 2004), whilst in this study the exposure intensity was 1140 mg MDI/m³ x min. In naïve BN-rats the concentration of protein in BAL was approximately twice that of the control at ~3000 mg MDI/m³ x min. In a previous study using essentially a similar design of elicitation, histopathological evidence of significant irritant effects was not provided at this exposure concentration and repeated challenge protocol. Therefore, the challenge concentration x time relationship chosen appears to be in the range of the irritation threshold, including the time spacing between each challenge, is believed to be suitable to differentiate unequivocally between irritant and immunologically related responses.

With regard to the transient respiratory signs suggestive of a "rhinitis"-like response observed after challenge it can be concluded that all challenges were tolerated without specific effects in the respective naïve control group. From the respective data shown in the Appendix it is apparent that the range of MDI concentrations at challenge 1 to 4 were ~37, ~34, 36-46, and 33-41 mg/m³, respectively. Therefore, the higher incidence of respiratory responses observed following the third challenge coincided with the concentration of MDI-aerosol. In MDI-sensitized animals the shape of the delayed-onset response shifted from mild, more prolonged effects to more rigorous excursions in Penh peaking approximately 3 hours after challenge. From Fig. 5 it appears that some immediate response might have occurred which is consistent with the clinical observations. Thus, with increasing number of challenge exposures the breathing patterns appear to shift to more "immediate-type" dual responses. The unifying principle to combine these types of changes is the AUC of Penh.

Results from bronchoalveolar lavage analysis and obtained by breathing pattern analysis for approximately 20 hours post-challenge were suggestive of an

unequivocal asthmagenic response. This was indicated by changes in BAL and included elevated protein, increased numbers of neutrophilic and eosinophilic granulocytes and total cell counts. Lung weights (absolute and relative) and lung-lymph-node weights were increased. Despite the marked differences in dosages and surface areas utilized to sensitize the animals, throughout the groups sensitized to and challenged 4-times with MDI, the magnitude of responses was essentially similar. Most changes gained statistical significance when compared with the non-sensitized and re-challenged control group. Lung weights and BAL endpoints of non-sensitized/re-challenged and non-sensitized/not-challenged were not at variance, supporting the hypothesis that the concentration regimen used for challenge was not associated with irritant-related confounding effects. Total IgE determinations did not reveal statistical differences amongst the groups. Animals induced topically (694 mg MDI/kg bw) and challenged approximately 3 months later displayed a borderline increase of indicators suggestive of an MDI-mediated asthmagenic response.

In summary, the findings of this study support the conclusion that this model in Brown Norway rats is suitable to identify MDI as an asthmagenic agent upon moderate to high topical induction dosages followed by repeated inhalation challenge exposures to mildly irritant concentrations of MDI. Consistent and unequivocally positive delayed-type changes of breathing patterns were observed in sensitized rats. These findings suggest that MDI promotes a more delayed-onset type rather than immediate-type inflammatory response. However, there are limitations with regard to the current dosing procedures used for the sensitization of animals as the total dose administered is undoubtedly linked to the surface area. This means both variables are interrelated. Accordingly, is appears to be difficult to disentangle unequivocally the role of 'total dose' *versus* 'surface area dose' in this animal model.

10. KEY TO ABBREVIATIONS IN TABLES

ECD ..... Effective cut-off diameter
STAND, S, Std, SD ..... Standard deviation (σ)
MW/MEANS, x ..... Means
B.W. ..... Body weight
F ..... F test value (F ratio)
DF ..... Degrees of freedom
PROB ..... Probability
SS ..... Total sum of squares
MS ..... Mean squares
TREATMENT ..... - Between the groups
ERROR ..... - Within the groups
TOTAL ..... - Total

11. REFERENCES

ACGIH (American Conference of Governmental Industrial Hygienists) (1978). Air Sampling Instruments for Evaluation of Atmospheric Contaminants, 5th Edition, ACGIH p. F-6. ACGIH section I: Calibration of Air Sampling Instruments and section F: Aerosol Sampling for Particle-size Analysis.

ASTM - Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals. ASTM Designation: E 981-84. American Society for Testing and Materials, Philadelphia, USA.

BCTIC Computer Code Collection - Biomedical computing Technology Information Center, ANOVA a Fortran Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville Tennessee, U.S.A.

Dennis, R (1976). Handbook of Aerosols - Technical Information Center, Energy Research and Development Administration, S. 110-114, July 1976.

EG Guideline 86/609/EC (1986). Guideline of the Council dated November 24, 1986 on the Reconciliation of Legal and Administrative Regulations of the Member Countries for the Protection of Animals used for Studies and other Scientific Purposes. Journal of the European Community, Legal Specifications L 358, 29.

Gad, SC and Weil, CS (1982). Statistics for Toxicologists. Principles and Methods of Toxicology, ed. A.W. Hayes, Raven Press, New York, p. 280.

Greenspan, L (1977). Humidity Fixed Points of Binary Saturated Aqueous Suspensions, Journal of Research of the National Bureau of Standards, Vol. 81 A, no. 1, Jan.-Febr. 1977.

Marple, VA and Rubow, KL (1980). Aerosol Generation Concepts and Parameters in Generation of Aerosols and Facilities for Exposure Experiments, Ed. K. Willeke, Ann Arbor Science Publ. Inc. Mich., pp. 3-29.

McFarland, HN (1976). Respiratory Toxicology - Essays in Toxicology, Vol. 7, pp. 121-154, Academic Press Inc., New York, San Francisco, London.

Pauluhn J (1997). Assessment of Respiratory Hypersensitivity in Guinea Pigs Sensitized to Toluene Diisocyanate: Improvements on Analysis of Response. Fundam. Appl. Toxicology 40: 211-219.

Pauluhn J, Eben A. (1991). Validation of a non-invasive technique to assess immediate or delayed onset of airway hypersensitivity in guinea-pigs. J. Appl. Toxicol. **11:** 423-431.

Pauluhn, J (1994). Validation of an improved nose-only exposure system for rodents. Journal of Applied Toxicology, 14:55-62.

Pauluhn J., Eidmann P., Freyberger A., Wasinska-Kempka G., Vohr H.-W. (2002). Respiratory Hypersensitivity to Trimellitic Anhydride in Brown Norway Rats: A comparison of endpoints. *J Appl Toxicol* 22: 89-97.

Pauluhn, J (2004). Comparative analysis of pulmonary irritation by measurements of Penh and protein in bronchoalveolar lavage fluid in Brown Norway rats and Wistar rats exposed to irritant aerosol. Inhalation Toxicology 16: 159-175.

Raabe, OG (1982).Deposition and Clearance of Inhaled Aerosols in H.Witschi and P.Nettesheim - Mechanisms in Respiratory Toxicology Vol.I, pp. 27-76, CRC Press, Inc. Boca Raton, Florida.

Snipes, MB (1989). Long-Term Retention and Clearance of Particles Inhaled by Mammalian Species. Critical Reviews in Toxicology, Vol. 20, pp. 175-211.

SOT-COMMENTARY (1992). Recommendations for the Conduct of Acute Inhalation Limit Tests, prepared by the Technical Committee of the Inhalation Specialty Section, Society of Toxicology. Fundam. Appl. Toxicol. 18, pp. 321-327.

USP XXII (1992). The United States Pharmacopeia, 22nd revision, Suppl. 7, NF XVII, Physical Tests and Determinations pp. 3122 - 3129.