FINAL REPORT

Laboratory Animal Allergy
Development of RAST Inhibition Assay
to Monitor Airborne Allergen Levels

Daniel M. Lewis, Ph.D.

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Project Officer: Daniel M. Lewis
Immunology Section, LIB, DRDS, NIOSH

Summary:

The objective of this project was to develop an assay that could be used to monitor airborne levels of allergens in the workplace. A cooperative agreement with the National Institute of Environmental Health Sciences (NIEHS) was developed to study the levels and distribution of rat derived allergens in the NIEHS facility in Research Triangle Park, North Carolina. This facility uses a large number of rats in their toxicology and research programs, and allergy to rats has become a problem for workers at NIEHS. The assay developed, the radioallergosorbent (RAST) inhibition assay, utilized sera from workers containing high levels of IgE antibodies to rat allergens to detect the allergens in extracts of dust samples. Development of the RAST inhibition assay involved screening serum samples from exposed workers to identify subjects with high IgE titers to the rat allergens, using a pool of those sera to develop optimal conditions for the RAST inhibition assay, using the RAST inhibition assay to evaluate sample collection and extraction procedures, and finally "field testing" the assay using airborne dust samples collected at the NIEHS facility. Two sets of samples were collected; one using personal samplers (which operate at relatively low flow rates) and one using high volume area samplers. The results demonstrated that the assay was sensitive enough to detect allergens on filters from personal samplers, but that the high volume area samplers worked better in that all samples collected had detectable allergen levels and that the ratio of total allergen level to total dust level was more consistent. These results confirmed that the assay could be reliably used for analysis of airborne allergen levels in the workplace. The results of these investigations are summarized in attachment 1.

During the course of these studies it became apparent that better characterization of the rat allergens was needed in order to identify the major source of the allergens (skin or urine), and to determine the cross-reactivity of rat allergens with other species of rodents. Cross-reactivity studies were performed using three different techniques; inhibition assays, cross absorption assays, and biochemical analysis of the extracts. The results of these studies showed that rat urine and epithelial extracts share antigens, but each contain unique antigens as well. In addition, cross reactions between rat and mouse derived antigens were detected, but not between rat and guinea pig or mouse and guinea

pig antigens. The finding that rat urine and epithelial antigens can be detected separately implied that control technologies should be design to control both sources of antigens. In addition, the cross-reactivity between rat and mouse antigens implies that exposure to one species may sensitize a person to both, and that the analysis of air samples may be complicated by the presence of both species within a research facility. These results are summarized in attachments 2 and 3.

In the cooperative agreement with NIEHS we had agreed to analyze samples collected at the University of Iowa where workers would be exposed to a variety of species of laboratory animals. When this cooperative agreement officially ended (Sept '89), we had not received the samples from the University of Iowa needed to complete that aspect of the study. Those samples have been obtained and analyzed. For these samples RAST inhibition assays for hamster and rabbit allergens were developed and used along with the assays for the three rodent species mentioned above. The results demonstrated that multiple allergens could be detected on a single filter. In addition, a prospective study of the development of IgE antibodies to laboratories animal was begun as part of the cooperative agreement. These studies are continuing as part of the agricultural initiative, and will require five years to complete. Publication of these results is anticipated when the study is completed.

Finally, the literature review done during the development and execution of this project served as a basis for a portion of a review paper that was recently published (attachment 4). This was a review of the guidelines for investigation of occupational asthma and included a discussion of environmental assessment using immunochemical techniques to detect airborne allergens.

Attachment 1

Scand J Work Environ Health 14 (1988): suppl 1, 74—76

Laboratory animal allergies

Use of the radioallergosorbent test inhibition assay to monitor airborne allergen levels

by Daniel M Lewis, PhD,[1] Toni A Bledsoe, MS,[1] John M Dement, PhD[2]

Occupational exposure to laboratory animals frequently leads to the development of allergies in exposed workers. The incidence of laboratory animal allergies has been estimated to be between 11 and 30 % of the exposed workers in the United States (2, 3). Occupational allergies can have a significant effect in terms of lost time at work and can even cause people to change positions and/or vocations. Although numerous studies have documented the prevalence of occupational allergies in general, and laboratory animal allergies in particular, less is known about the environmental factors which influence the development and expression of these diseases. The current study was undertaken to develop methods of monitoring airborne levels of allergens so that environmental controls could be applied to prevent or lessen allergic reactions in workers exposed to laboratory animals. Previous studies have been reported in which airborne levels of mouse and guinea pig allergens were measured with a modification of the radioallergosorbent test (RAST), termed the RAST inhibition assay (4, 5). Although these studies demonstrated that the RAST inhibition assay could be used to quantify airborne allergen levels, they were performed using only area sampling techniques. We have investigated the utility of this assay to detect airborne rat allergen levels by both personal and area sampling, and we have attempted to make a better delineation of the rat allergens in the air.

Materials and methods

Extracts of rat epithelium and urine were prepared as previously described (5). Briefly, for epithelial extracts rat pelts were lyophilized, defatted by ether extraction in a Soxhlet apparatus, ground in a Wiley mill, and extracted as a 5 % suspension in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. The extract was dialyzed against 0.05 M ammonium bicarbonate, lyophilized, and stored at 4°C until used. The urine extracts were prepared by dialysis against 0.05 M ammonium bicarbonate buffer, filtration through a 0.45 μ porosity filter, and lyophilization. The extracts were coupled to cyanogen bromide activated Sepharose-4B beads according to the manufacturer's recommendations (Pharmacia, Piscataway, New Jersey). The extracts used in the inhibition assay were dissolved in saline at an epithelial protein concentration of 10 mg/ml and a urinary protein concentration of 20 mg/ml, as determined by the BioRad protein assay (BioRad Laboratories, Richmond, California).

Air samples were collected with DuPont personal sample pumps at a flow rate of 2 l/min and type FWS-D polyvinyl chloride membrane filters. The filters were extracted in 10 ml of 0.05 M ammonium bicarbonate, and the extract was lyophilized and redissolved in 1.0 ml of sterile saline.

The RAST procedure was performed as described (6) with the use of 50 μl of a 10 % suspension of the allergen-coupled Sepharose-4B beads per tube and iodine-125-labeled antihuman immunoglobulin E (IgE) obtained from Pharmacia Diagnostics. The RAST procedure was used to screen the sera from workers exposed to rats and identify those with high levels of IgE antibodies to rat allergens. Sera from five individuals were pooled and used as the reference serum for the RAST inhibition assay. The reference serum pool contained 160 IU/ml of IgE, and a 1:5 dilution of the pool would bind approximately 15 % of the radioactivity in the standard RAST assay.

The RAST inhibition assay (1) was performed using 200 μl of either the reference allergen or air sample extract mixed with 250 μl of diluted reference serum and 50 μl of allergen-coupled beads, the volume was brought up to 900 μl with sample diluent (0.1 M phosphate buffer, pH 7.4, containing 4 % fetal calf serum, 1 % Tween 20, and 0.01 % NaN_3), and the mixture was incubated overnight at room temperature on a tube rotator. The tubes were washed three times, radiolabeled anti-IgE was added, and the tube was incubated overnight at room temperature. After three additional washes, the radioactivity of the samples was determined in a gamma counter. The percentage of inhibition was calculated for each sample, and a standard curve was made by the plotting of the logit of the percentage of inhibition against the concentration of protein in the reference samples. By regression analysis the allergen content of each sample was calculated and

[1] Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA.
[2] Health and Safety Office, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA.

expressed as micrograms of protein allergen per cubic meter of air.

Results and discussion

Initial studies were conducted to determine if the extracts of rat allergens coupled to the beads contained the appropriate allergens. For this purpose, sera from 36 individuals occupationally exposed to rats were assayed with the use of the reagents prepared in our laboratory and the commercially available RAST reagents. There was complete concordance between these assays in that all the subjects were either negative in both assays or positive in both assays. In addition, with IgE-positive subjects the relative reactivity (percentage of counts bound) was comparable in both assays (data not shown). The results from these assays were used to identify five individuals who had relatively high reactivity in the RAST, additional serum was obtained from these subjects, and a reference serum pool was established.

A series of experiments was carried out to determine the optimal conditions for the RAST inhibition assay. The volume of each of the reactants and the incubation times and conditions were determined which gave the best results. As shown in figure 1, a linear relationship between the logit of the percentage of counts bound and the protein content of the extracts was obtained, correlation coefficients (r) of 0.97 or better typically being obtained. The lower limits of detection were estimated to be 0.6 µg/ml for the urinary allergens, and 1.4 µg/ml for the epithelial allergens.

Air samples were collected from a variety of sites and assayed for allergen content. In figure 2 the concentration of urinary and epithelial allergens have been plotted against each other. There were several samples in which only one of the allergens was detected. In addition the airborne levels of urinary and epithelial allergens did not correlate ($r = 0.36$). This finding would suggest that there are antigens that are unique to each extract. When these data were analyzed with respect to the site of sample collection, the results were generally as expected. In other words, areas with the highest allergen levels were areas with either many animals (eg, holding rooms) or a high dust exposure level (eg, cage dumping and cleaning areas). Of interest was the finding of detectable levels of the allergens in the locker room and work break room, ie, in the rooms where employees changed their clothes or took work breaks. These results suggest that the allergens may be carried on clothing and distributed throughout the worksite.

In order to define the allergens contained in the extracts better, a sample of the urine extract was chromatographed on a Sephacryl S-300 column, and the major protein fractions were assayed for allergen content. As shown in figure 3, the allergenic activity eluted in two peaks indicating that the urine contained at least two allergens. A sample of the epithelial extract was

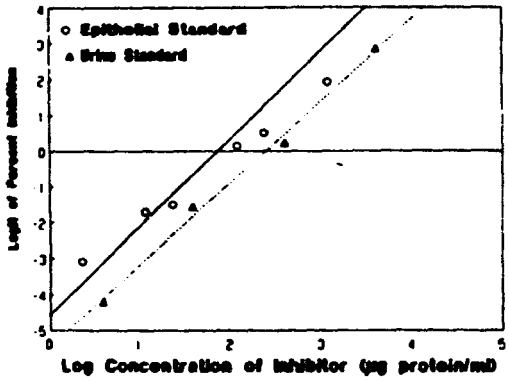

Figure 1. Linear relationship between the logit of the percentage of counts bound and the protein control of the extract.

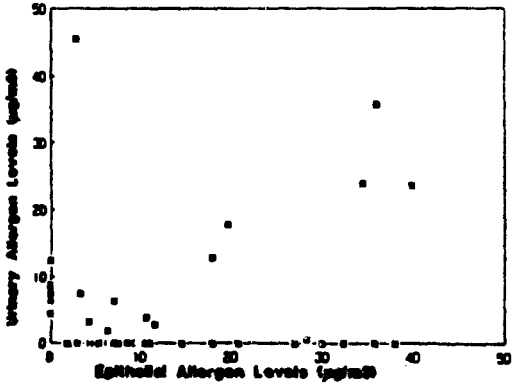

Figure 2. Concentration of urinary and epithelial allergens plotted against each other.

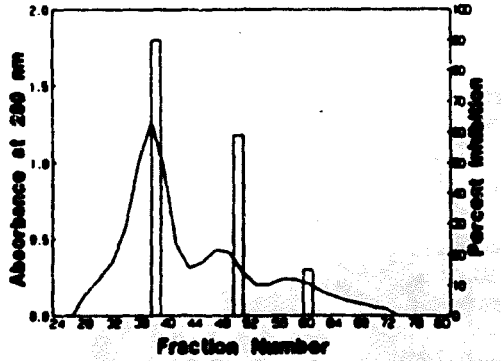

Figure 3. Chromatograph of the urine extract showing the major protein fractions.

chromatographed, and the allergenic activity was found to be widely distributed in the column eluate. This finding indicates that the epithelium is a much more complex mixture of allergens than the urine. It is hoped that, by using more purified allergens, we will be able to increase the sensitivity and specificity of the RAST inhibition assay.

In summary, the results obtained to date indicate that the RAST inhibition assay provides a very sensitive method to detect airborne allergens of laboratory animals. The use of serum from workers allergenic to rats in this assay assures the specificity of the assay. We have found that, by concentrating filter extracts by lyophilization, we can detect as little as 1 μg of allergen on a filter and thus allow for the use of personal samplers to monitor the work environment. Even though we are continuing to attempt to improve the sensitivity of the assay, the assay has proved to be very reliable and consistent. Quantitative assessment of the airborne allergen levels in the workplace should allow for the development of control measures to reduce allergen levels either by changes in work practices or by engineering controls.

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Attachment 2

Major Rat Allergens Causing Laboratory Animal Allergies

T. Bledsoe, M.S., (ASCP)* and D. Lewis, Ph.D.
National Institute for Occupational Safety and Health
Morgantown, WV

Occupational allergies can result in lost time at work and a change in position or vocation by the employee. Studies concerning the prevalence of laboratory animal allergies (LAA) have estimated the incidence of LAA among animal care workers to be eleven and thirty per cent. The rat is the most frequently reported cause of LAA (72.2%) and this study identifies the antigenic components of two major sources of rat protein: rat urine and rat epithelia. Four epithelial protein fractions and three urinary protein fractions were identified by column chromatorgaphy. These fractions were determined to have marked antigenic activity by the radioallergosorbent (RAST) inhibition assay. The results demonstrated that the rat urinary and the rat epithelial extracts had similiar antigenic components but also each had antigenic components unique to itself.

ABSTRACT

Occupational allergies can result in lost time at work and a change in position or vocation by the employee. Studies concerning the prevalence of laboratory animal allergies (LAA) have estimated the incidence of LAA among animal care workers to be between eleven and thirty percent. The rat is the most frequently reported cause of LAA (72.2%) and this study identifies the antigenic components of two major sources of rat protein: rat urine and rat epithelia. Four epithelial fractions were identified by column chromatography. These fractions were determined to have marked antigenic activity by the radioallergosorbent (RAST) inhibition assay. The results demonstrated that the rat urinary and the rat epithelial extracts had similar antigenic components but also each had components unique to itself.

INTRODUCTION

- It is estimated that thirty-five thousand animal care workers, laboratory technicians, scientists, and veterinarians in the United States are regularly exposed to lab animals.
- The incidence of laboratory animal allergies (LAA) in the United States has been estimated to be between eleven and thirty percent, or one in five exposed workers.
- The majority of the exposed workers become symptomatic between six and twenty-four months of workplace exposure.
- LAA can be defined as an allergic disorder generally charactorized by rhinitis and conjunctivitis but may also be accompanied by rash, urticaria, asthma, and cough.
- Five species comprise 99% of lab animals: rats, mice, guinea pigs, rabbits, and hamsters.
- The number of types of animals to which workers are exposed generally correlate with the probability of LAA.
- The rat is the most frequently reported cause of LAA (72 %).
- Sources of allergens are in shed dander, hair, saliva, serum, urine, and tissues (i.e. epithelial).

LABORATORY ANIMAL ALLERGENS STUDY

RAST Class	Guinea Pig	Hamster	Mouse	Rabbit	Rat
4	0	0	1	1	1
3	0	1	0	0	4
2	2	1	3	5	3
1	0	0	1	0	4
1/0	2	2	0	1	4
0	136	136	135	133	128
% Positive =	2.9%	2.9%	3.6%	5.0%	8.6%

140 serum samples from the University of Iowa were screened for specific IgE activity to the above species.

Rat was the most frequent cause of LAA (8.6%).

METHODS

This study identifies the antigenic components of rat epithelia and rat urine by the following procedures:

1. Epithelial Extract Preparation
2. Urinary Extract Preparation
3. Column Chromatography
4. SDS Acrylamide Gel and Two-Dimensional Electrophoresis
5. RAST Inhibition: using pooled RAST Class 4 rat sensitive sera.

EPITHELIAL EXTRACT PREPARATION

Pelts: Freeze-dried and Defatted

Ground with Wiley Mill

Extracted 24 hours in PBS, pH 7.4

Filtered through .80 um + prefilter, .45 um, and .20 um

Dialyzed vs. 0.05 M NH_4HCO_3

Centrifuged and Lyophilized

Redissolved in Bicarbonate Buffer, pH 8.3

Coupled to CNBr Sepharose-4B Beads

Stored at 4 C in Tris-HCl Buffer, pH 8.0 with Azide

URINARY EXTRACT PREPARATION

Urine: Centrifuged and Sediment discarded

Dialyzed vs. 0.05 M NH_4HCO_3

Centrifuged and Sediment discarded

Filtered through .80 um + prefilter, .45 um, and .20 um

Lyophilized

Redissolved in Bicarbonate Buffer, pH 8.3

Coupled to CNBr Sepharose-4B Beads

Stored at 4 C in Tris-HCl Buffer, pH 8.0 with Azide

RAST INHIBITION PROTOCOL

50 ul Beads + 250 ul 1:5 Diluted Serum + 200 ul Extract

Incubate overnight at room temperature on tube rotator

Wash three times with RAST Buffer

Counted in a gamma counter

Calculate Percent Count Bound and Percent Inhibition

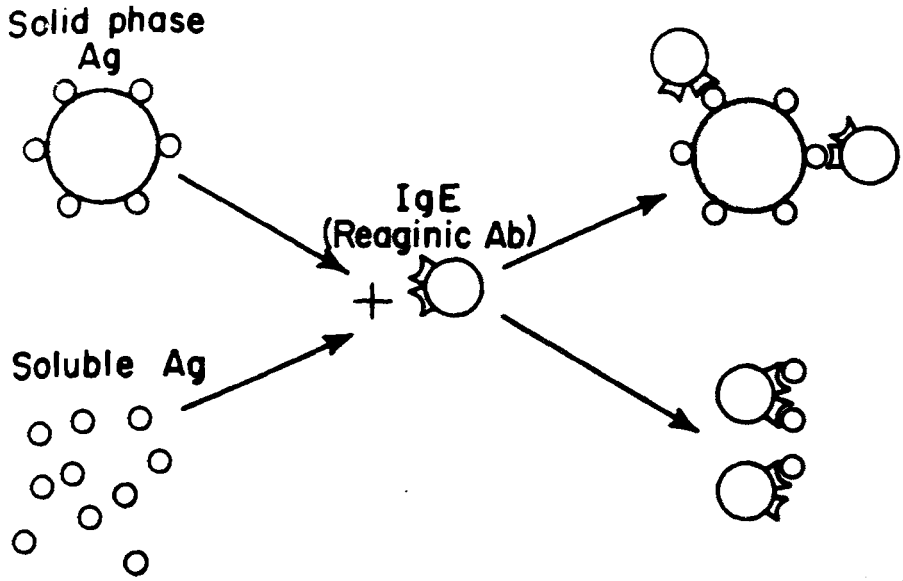
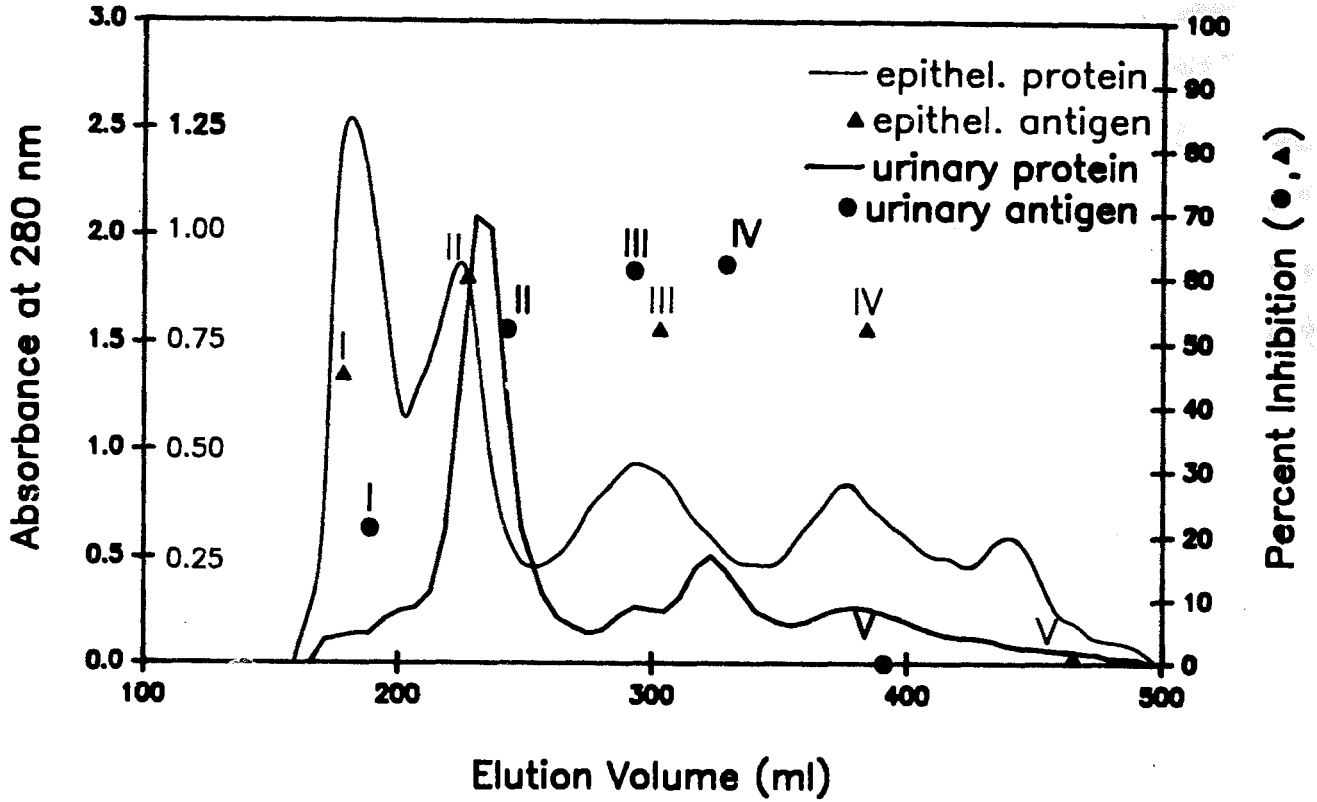


FIG. 1. Diagrammatic representation of the first stage of RAST inhibition procedure. There is competition between soluble and solid-phase allergens for the available IgE antibodies. Ag, Antigen; Ab, antibody. Used with permission from G. J. Gleich, J. B. Larson, R. T. Jones, and H. Baer, J. Allergy Clin. Immunol. 53:158–169. 1974.

EPITHELIAL & URINARY ALLERGEN EXTRACT



Chromatograph of epithelial & urinary extract showing major protein fractions & % inhibition for each fraction. % inhibition was not performed for Urin. Fraction V due to insufficient sample volume.

URINARY EXTRACT RESULTS

- Two peaks of maximum allergenic activity were seen in in the column effluent in the protein size ranges: 74 kd and 12-25 kd.
- The 3rd peak observed in the column effluent was divided into Fractions III and IV.
- Inhibition was observed as Urine Fraction I (21%), Fraction II (52%), Fraction III (61%), and Fraction IV (62%).
- Fractions III & IV contains protein in the size range of the rat urine major allergenic component: two partially cross-reacting forms: alpha-2 euglobulin (16-18 kd) & prealbumin (20-21 kd).

SDS Gel & Two-Dimensional Electrophoresis:

- Urine extract: observed protein concentrated in the 50-74 kd and 12-25 kd ranges.
- Fraction I: observed protein concentrated in the 50-74 kd range.
- Fraction II: observed protein concentrated in the 50-74 kd and 25 kd ranges.
- Fractions III & IV: observed protein concentrated in the 12-25 kd range increased and protein concentrated in the > 25 kd range decreased.

EPITHELIAL EXTRACT RESULTS

- Four peaks of maximum allergenic activity were seen in the column effluent in the protein size ranges: > 67 kd (kilodalton), 67 kd, 25 kd, and < 13 kd.
- Inhibition was observed as Epithel. Fraction I (45%), Fraction II (60%), Fraction III (52%), and Fraction IV (52%).

SDS Gel & Two-Dimensional Electrophoresis:

- Epithelial Extract: observed proteins with mobility similar to a gamma protein with migration toward the (+) pole.
- Fraction I: observed protein concentrated in the > 74 kd, 37 kd, and 25 kd ranges.
- Fraction II: observed protein concentrated in the 74 kd range increased.
- Fraction III: observed protein concentrated in the 12-25 kd and the < 12 kd ranges increased.
- Fraction IV: protein bands not detected by SDS or two-dimensional electrophoresis; protein concentration may have been below method detection limit or protein did not migrate toward the (-) pole (gamma protein) .

SUMMARY

- The cross-sectional lab animal allergy study illustrated rat allergens as the most prevalent cause of lab animal allergies among animal care workers.
- Both rat epithelia and rat urine exhibited major allergenic activity in the protein size ranges of 67 kd (albumin) & 12-25 kd (prealbumin & alpha-2 euglobulin).
- Urine III & IV demonstrated the greatest allergenic activity (61% & 62% I) and contained protein in the size range of rat urine major allergenic component: prealbumin and alpha-2 euglobulin.
- Epithelial extract demonstrated the presence of protein with mobility similar to gamma protein.
- Rat epithelia demonstrated widely distributed allergenic activity while urine allergenic activity was primarily limited to two size ranges.
- Rat epithelia and rat urine contain similar protein components but epithelia appears to be more complex.

Attachment 3

CROSS-REACTIVITY OF LABORATORY ANIMAL ALLERGENS: INTER AND INTRA SPECIES STUDIES

Lewis, Daniel M. [1]
Bledsoe, T.A. [1]
[1] National Institute for Occupational Safety and Health, Morgantown, USA

OBJECTIVE: The objective of this study was to determine the cross-reactivity of allergens prepared from the pelts and urine of rats and mice. Partially purified allergens from rat urine and pelts were analyzed to determine if there were antigens unique to each source.

METHODS: Aqueous extracts of freeze-dried, defatted pelts of rats and mice were prepared as a source of epithelial allergens, and dialyzed urine was use as a source of urinary allergens. The allergens were coupled to cyanogen bromide activated Sepharose 4B beads (solid phased allergens), or diluted in phosphate buffer containing 1% bovine serum albumin and 0.1% Tween 20 (soluble allergen). Cross-reactivity was assessed by radioallergosorbent test (RAST) inhibition assay, and by cross absorption studies.

RESULTS: Using a pool of sera from workers primarily exposed to rats we found that the sera reacted equally well in a RAST assay with rat urine and epithelial allergens and with mouse urine epithelial allergens. To determine if this was due to cross-reacting antibodies, RAST inhibition studies were preformed. The rat epithelial and urine antigens were weakly cross reactive with mouse epithelial antigens, but not with the mouse urine antigens. Similar studies comparing rat urine and epithelial allergens with each other revealed that there was a high degree of cross-reactivity. Cross absorption studies with solid phased urine or epithelial allergens did not completely remove cross reacting antibodies. To better determine if the urine and epithelial extracts contained unique as well as common antigens, the extracts were chromatographed on Sephacryl S-200 and the various fractions tested in RAST inhibition. The results showed that there are antigens common to both urine and epithelium, and antigens unique to each source.

CONCLUSIONS: In an earlier study of the airborne distribution of allergens within a research facility we found that rat urine and epithelial allergens could be detected separately, and that sera from exposed workers contained similar levels of IgE antibodies to both types of rat and mouse allergens. The results of this study indicate that there is cross-reactivity between rat and mouse epithelial allergens, and thus exposure to one of these species may lead to sensitization to both. In addition we found that while rat urine and epithelium share antigens there are antigens unique to each source. This finding helps explain our earlier observation that the airborne distribution of rat allergens is different for epithelial and urinary allergens.

INTRODUCTION

Occupational exposure to laboratory animals is frequently associated with the development of allergies. The prevalence of allergy to laboratory animals has been estimated to be between 11 and 30% among animal caretakers and laboratory workers. Previous studies which have evaluated the prevalence of allergy to particular species of laboratory animals have not yielded consistent results, but laboratory rodents; rats, mice and guinea pigs; are frequently identified as problems. Within these species the urine and epithelium (pelt) are the major sources of allergenic proteins.

In earlier studies we found that sera from workers primarily exposed to rats contained IgE antibodies to rat and mouse epithelial and urine allergens. To determine if these distinct or cross-reactive antibodies, we investigated the allergens of rats, mice, and guinea pigs to determine both the cross-reactivity between species and between the urine and epithelial derived allergens.

TABLE 1

RAST Analysis of Selected Sera

Serum * Source	Allergen** GP Epi	GP Ur	M Epi	M Ur	R Epi	R Ur
Serum Pool	4.6***	4.3	11.5	10.0	11.2	12.9
Subject 1	10.1	8.2	3.6	4.1	9.6	7.9
Subject 2	17.4	6.5	21.3	18.3	27.2	24.0
Subject 3	5.2	nd	11.8	10.1	14.6	11.8

* - Serum pool was from five rat sensitive individuals whose primary exposure was to rats.

Subject 1 was a guinea pig sensitive subject who avoided guinea pigs but did have exposure to rats. Subjects 2 and 3 had frequent exposures to both rats and guinea pigs but not mice.

** - Abreviations: GP Epi = guinea pig epithelial antigen
GP Ur = guinea pig urine antigen
M Epi = mouse epithelial antigen
M Ur = mouse urine antigen
R Epi = rat epithelial antigen
R Ur = rat urine antigen

*** - Results expressed as RAST Score which was calculated by dividing the percent counts bound by antigen coated beads by the percent counts bound by HSA coated beads.

CONCLUSION: Sera from workers whose primary exposures were to rats and guinea pigs had comparable levels of IgE antibodies to mice allergens. These results suggest that the antibodies were cross-reactive to rodent allergens.

RAST Inhibition Protocol

50 μl beads + 250 μl serum + 200 μl extract

Incubate overnight at room temp.
on tube rotator

Wash 3X with RAST buffer

Add I-125 anti-IgE

Incubate overnight

Wash 3X with RAST Buffer

Count in gamma counter

Calculate percent counts bound
and percent inhibition

Mouse Cross Reactivity Studies

Inhibitor	Mouse Urine	Mouse Epitheliun
Mouse Urine	260*	no inhibition
Mouse Epithelium	10,000	271
Rat Urine	114	526
Rat Epithelium	3185	68
G.P. Urine	no inhibition	no inhibition
G.P. Epithelium	no inhibition	no inhibition

* - 50% Inhibition Concentration (ug protein/ml)

Conclusion: Guinea pig antigens did not cross react with mouse antigens, but the rat derived antigens did. Of note the mouse epithelial and urine antigens did not cross react significantly.

Guinea Pig Cross Reactivity Studies

Inhibitor	G.P. Urine	G.P. Epithelium
G.P. Urine	110*	21
G.P. Epithelium	1585	10
Rat Urine	no inhibition	no inhibition
Rat Epithelium	no inhibition	4400
Mouse Urine	no inhibition	no inhibition
Mouse Epithelium	no inhibition	no inhibition

* - 50% Inhibition Concentration (ug protein/ml)

Conclusion: Rat and mouse antigens do not appear to cross react with guinea pig derived antigens. The guinea pig epithelial and urine antigens do appear to cross react.

Rat Cross Reactivity Studies

Inhibitor	Rat Urine	Rat Epithelial
Rat Urine	141*	71
Rat Epithelium	267	2470
Mouse Urine	no inhibition	no inhibition
Mouse Epithelium	6671	no inhibition
G.P. Urine	no inhibition	no inhibition
G.P. Epithelium	no inhibition	no inhibition

* - 50% Inhibition Concentration (ug protein/ml)

Conclusion: The mouse and guinea pig antigens did not cross with the rat derived antigens significantly. The rat epithalial and urine antigens do appear to cross react.

Cross Absorption Protocol

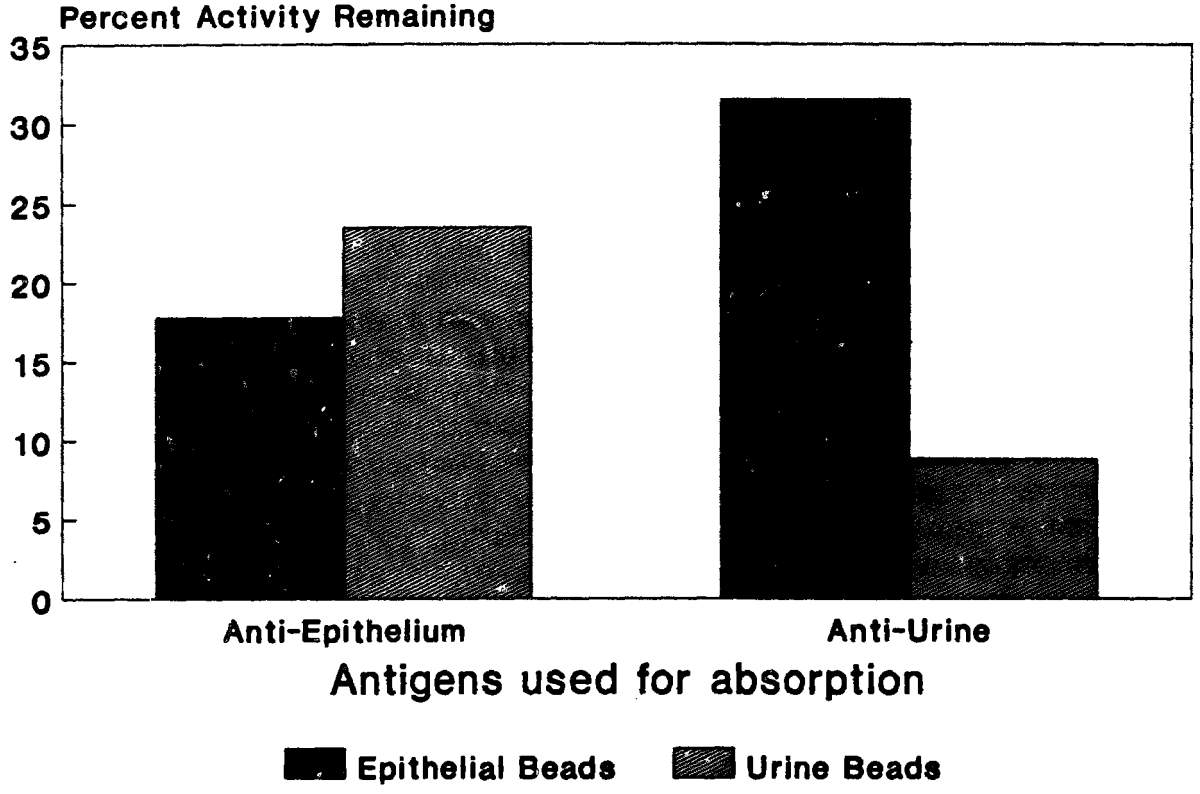
1.0 ml serum + allergen coated beads
Incubate overnight

Recover serum

Perform direct RAST with serum

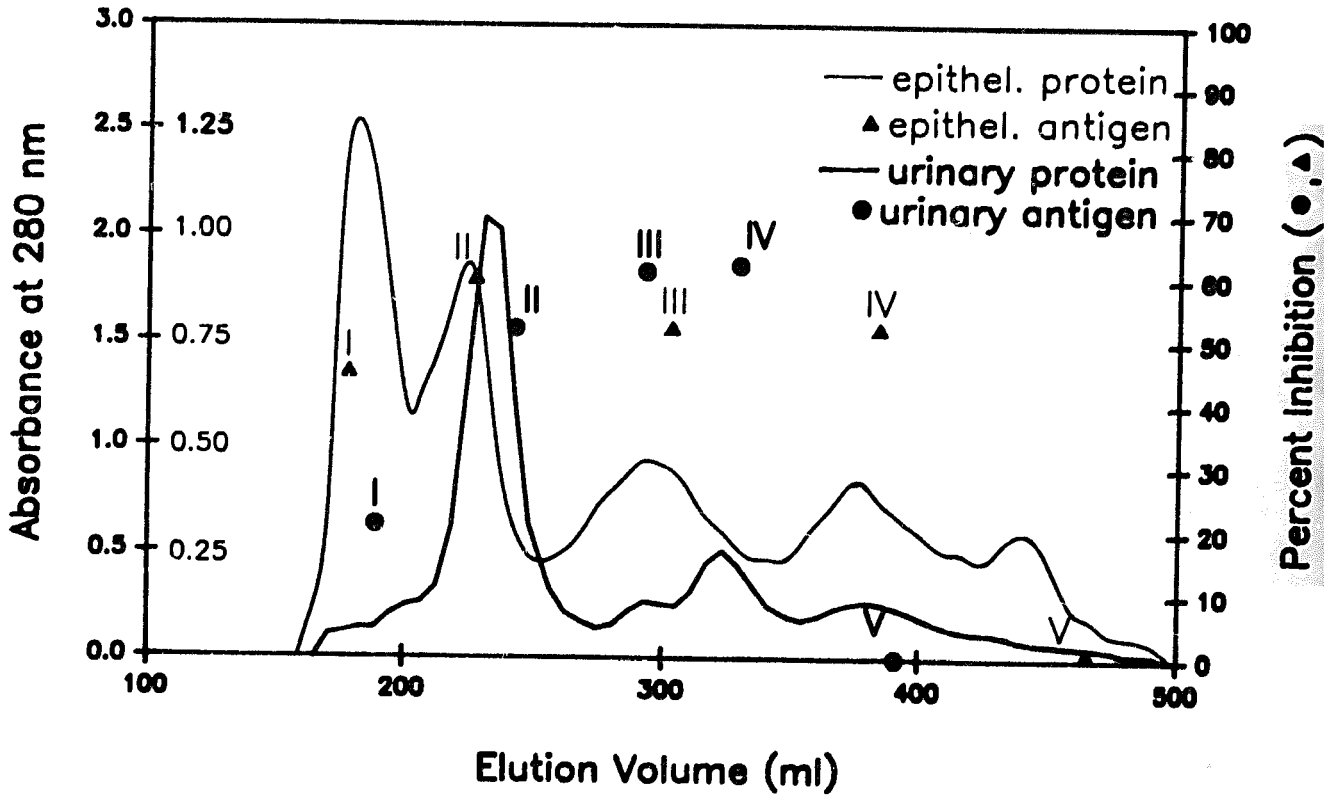
Absorbants used:
Rat urine coated beads
Rat epithelium coated beads
Human serum albumin coated beads

Cross Absorption Studies



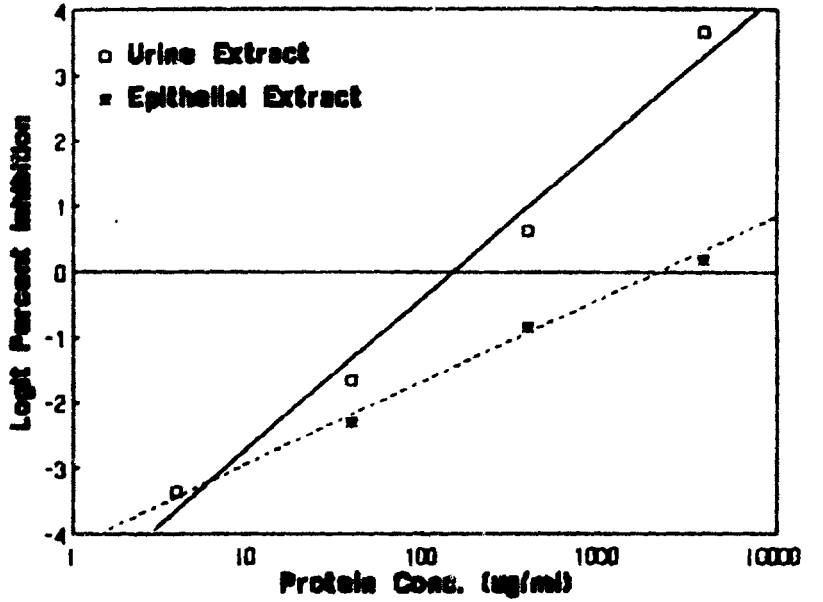
Conclusion: Results indicate that epithelial and urine beads were equally effective in absorbing antibodies to epithelial antigens, but epithelial beads were less effective in absorbing anti-urine antibodies.

EPITHELIAL & URINARY ALLERGEN EXTRACT

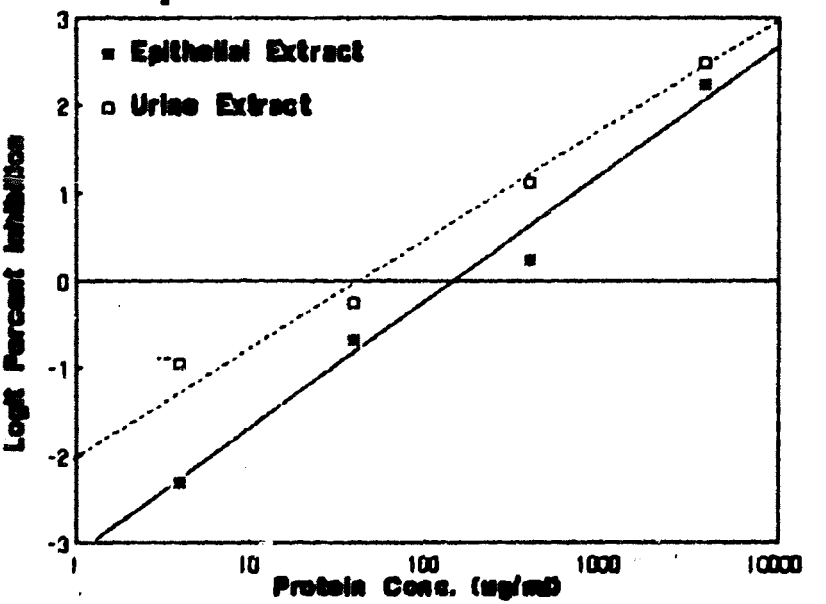


Chromatograph of epithelial & urinary extract showing major protein fractions & % inhibition for each fraction. % inhibition was not performed for Urin. Fraction V due to insufficient sample volume.

Urine Standard Curve



Epithelial Standard Curve



Studies of the Cross Reactions of Rat Urine and Epithelial Antigens

Conclusion: Comparison of the standard curve made using either rat urine or rat epithelial beads shows that with epithelial beads parallel lines of inhibition are obtained with both type of antigen. However, using urine beads the inhibition curves are not parallel. This suggests that the urine contains antigens not found in the epithelial antigen extract.

CONCLUSIONS

The results of this study indicate that there is cross reactivity between rat and mouse allergens. Thus exposure to one of these species may lead to sensitization to both. The guinea pig allergens do not appear to cross react with either rat or mouse allergens.

The analysis of the cross reactivity of rat urine and epithelium reveals that while there are antigens common to both, the urine contains antigens not found in the epithelium. The gel filtration studies suggest that the epithelium contains antigens not found in the urine, but the cross absorption studies do not confirm this.

Attachment 4

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Guidelines for the Epidemiologic Assessment of Occupational Asthma

Report of the Subcommittee on the Epidemiologic Assessment of Occupational Asthma, Occupational Lung Disease Committee

Alexander Blair Smith, MD,* *Chairperson*, Robert M. Castellan, MD, Dan Lewis, PhD,** and Thomas Matte, MD*****

Physician reporting of cases of communicable disease has played an important role in detection and control of disease outbreaks.[1] A similar expectation exists for physician reporting of occupational diseases, including occupational asthma. The diagnosis of a single case of occupational asthma may be a sentinel event that reveals the need to search for other cases, as well as remediable underlying causes.[2] As stated by Rutstein et al.,[3] such an occurrence may: "(1) provide the impetus for epidemiologic or industrial hygiene studies, or (2) serve as a warning signal that materials substitution, engineering control, personal protection, or medical care may be required." Also, other purposes may be served by consequent epidemiologic and industrial hygiene studies. An epidemiologic survey may be needed to convince responsible parties that a health problem exists that is in some measure attributable to workplace exposures. An epidemiologic survey will be needed to quantitate the magnitude of a work-related health problem, whether the magnitude is expressed as disease prevalence, incidence, or some other measure. An epidemiologic study may identify personal risk factors that explain why certain persons have manifested a disease as a consequence of their occupational exposure(s). Resultant recommendations for control of exposures in the workplace may facilitate management of the index case and affected co-workers, and help prevent the occurrence of additional cases.

INITIAL INVESTIGATION

The epidemiologic study most suited to the initial investigation of suspected occupational asthma is that described by Miettinen[4] as the "census-sample" study. Here, the investigator first conducts a survey (or "census," in Miettinen's terminology) to determine a study population's disease experience or to identify the presence of some characteristic suggestive of the disease in question. The investigator then conducts a detailed survey or examination of persons identified as cases and of a sample of the underlying population to obtain further information on the personal and environmental correlates of the disease. If potential cases are identified by a characteristic suggestive but not diagnostic of the disease, results of appropriate diagnostic tests should reduce false-positive classification of potential cases that do not truly have the disease.

For example, in a study of occuaptional asthma, an initial survey might be conducted of all workers by questionnaire, to classify each respondent with respect to symptoms suggestive of asthma (i.e., episodic wheezing, chest tightness, or shortness of breath). A second survey would be conducted on all potential cases and a sample of the underlying population to ascertain additional information. This information might include physiologic tests that determine whether underlying symptoms are attributable to asthma and tests that examine the temporal relationship of symptoms to precipitating circumstances at work. Other occupational correlates may be used, such as tests that measure sensitization to general environmental antigens and measure potential workplace allergens. Although not specifically identified as such, this approach has been used in many published studies, although in some studies the second survey of a sample from the underlying population may appear to have been omitted.[5-7]

The "census-sample" approach appeals to common sense. The initial survey is used not to provide definitive diagnoses, but only to identify workers with symptoms that warrant further evaluation.[7] Best use is made of researchers' time and resources because they are not spent evaluating in detail a preponderance

From the *National Oceanic and Atmospheric Administration and the *Department of Family and Community Medicine, Eastern Virginia Medical School, Norfolk, Virginia, and the National Institute for Occupational Safety and Health, **Morgantown, West Virginia, and ***Atlanta, Georgia.

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TABLE I. Sensitivity and specificity of symptoms, as well as symptom-based definitions of asthma, for positive MTC

Symptom/definition	Sensitivity	Specificity
Wheeze, regardless of other symptoms	67/229 = 29%	991/1163 = 85%
Breathlessness, regardless of other symptoms	82/229 = 30%	964/1163 = 82%
Wheeze and breathlessness	39/229 = 17%	1107/1163 = 95%
Wheeze or breathlessness	110/229 = 48%	848/1163 = 73%
Any respiratory symptom	149/229 = 65%	680/1163 = 58%
Asthma diagnosis		
Brooks[25]	49/229 = 20%	1067/1163 = 92%
Cookson[26]	43/229 = 19%	1020/1163 = 88%
Any reported	28/229 = 12%	1131/1163 = 97%
Physician diagnosed	23/229 = 10%	1148/1163 = 99%

Adapted from Enarson DA, et al. Am Rev Respir Dis 1987;136:65.

used as the standard, leading the authors to state that "bronchial hyperresponsiveness in epidemiologic surveys is limited at present in identifying subjects with asthma."[20]

This lack of a uniformly accepted gold standard precludes measurement of the true sensitivity and specificity of any test for asthma. However, "sensitivity" and "specificity" are still commonly used in reporting comparisons between two imperfect tests, in which one is used as a proxy for the standard. A limited number of studies have specificed independent—albeit imperfect—operational standards for evaluating their questionnaires.

High sensitivity and specificity are both desirable in any test, but in many instances, high sensitivity compromises specificity and vice versa. Given the screening function that questionnaires serve in epidemiologic studies, high sensitivity should take priority in the design of questionnaires on occupational asthma. Unfortunately, limitations in study design or in the data reported often prevent a direct assessment of the sensitivity and specificity of respiratory symptoms as elicited by questionnaire. These limitations include a failure to apply the standard to a sample of those screening negative by the questionnaire and the use of an additional questionnaire to refine the initial screen.[1, 21, 22] Other studies are found in which the standard has incorporated to an unknown extent the results of the test to which it is being compared.[23] Finally, because working populations tend to differ from community populations in the distribution of relevant covariates and other diseases, evaluations of questionnaires in community populations may not predict their performance in worker groups.

In studies that compare symptoms elicited by questionnaire to more objective, independent correlates of asthma, investigators generally have chosen tests of nonspecific bronchial hyperresponsiveness (NSBH), such as methacholine inhalation challenge (MIC), as the standard against which the sensitivity and specificity of questionnaire items are assessed. Because NSBH is believed to be sensitive but not specific for identifying asthma, use of NSBH as a standard can result in underestimating the sensitivity of the questionnaire for the disease, as opposed to its sensitivity for NSBH as a surrogate for the disease.[24] Furthermore, the choice of criteria for a positive test of NSBH and the choice of questionnaire responses used to define occupational asthma will both influence apparent questionnaire performance.

Studies can be found in which questionnaire responses correlate well with bronchial hyperresponsiveness. However, the preponderance of the limited published evidence suggests that questionnaires function more poorly than an advocate for the "census-sample" study of occupational asthma would hope. Dales et al.,[19] using a French translation of the ATS-DLD questionnaire, studied 200 Canadian insulation workers. The sensitivity and specificity of "wheeze," "wheeze with dyspnea," and a "history of asthma," were assessed against methacholine challenge. A nonstringent criterion for a positive test was a PC_{15} (provocative concentration causing a 15% decline in FEV_1) of less than 16 mg/ml. The sensitivity of "wheeze" for a positive methacholine challenge was 26%, "wheeze with dyspnea" was 10%, and of a "history of asthma" was 7%. The specificities were 87%, 93%, and 97%, respectively. The sensitivity of "cough" and of "cough with sputum" was greater than that of "wheeze" (45% and 33%, respectively), with correspondingly lesser specificity. This latter observation suggested that the positive methacholine tests

struction have been most commonly used in epidemiologic studies of occupational asthma: standard spirometry performed before and after a work shift, and ambulatory monitoring of peak expiratory flow rates over a period of weeks.

Spirometry "shift" studies. In a modification of the "stop-resume work test" traditionally used to assess work-related allergy, acute work-related decrements in ventilatory function were first documented in an epidemiologic study by measuring spirometry just before and after a Monday work shift among cotton workers.[36] Since then, spirometric measurement of forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and their ratio have become the most commonly used and standardized tests of lung function, both in the clinical laboratory and in epidemiologic field studies. Standard spirometry is easily performed in only a few minutes and is very reproducible. The required instrumentation is relatively inexpensive and portable.[37] Much of the utility of the FEV_1 and FVC derive from understanding sources of variability and controlling those that might confound or obscure test results.

Variability of spirometry measurements results from measurement error and from true biologic variation.[12] Measurement error can be effectively minimized by consistently following generally accepted recommendations for standardization of spirometry instrumentation and procedures.[38] Thus, proper spirometry testing should include quality equipment, frequent calibration, leak checks, and BTPS correction factors to minimize measurement errors. At least three acceptable maximal expirations should be recorded for each subject, and these should be properly measured using standard methods. Quality assurance programs built into automated spirometers are no substitute for visual inspection of all spirograms for acceptability.[39] Spirometer temperatures should be carefully controlled at field study test sites to avoid the variable effect of spirometer temperature on BTPS-corrected FEV_1 results.[40, 41]

In addition to measurement errors attributable to instrumentation or technique, true diurnal variation in airflow may serve to obscure the airway effect of an exposure in the workplace.[42] Even with optimal spirometry testing, some amount of variability exists in repeated measures on the same individual in the absence of airway insult. Over short intervals of laboratory testing in relatively healthy subjects, mean intrasubject coefficients of variation for FVC and FEV_1 have generally been found to be on the order of 3% or less,[43] with only slightly greater values found under field testing conditions.[44] There is good evidence, however, that these parameters are not so reproducible among subjects with chronic "fixed" obstruction in which coefficients of variation for FEV_1 and FVC have been on the order of 10%.[45, 46] Understanding this residual variability is critical to an appropriate interpretation of spirometry results from "shift" studies. Therefore, arbitrary exclusion of spirometry data that do not meet recommended reproducibility standards may bias results.[47] This is particularly true in short-term longitudinal studies used to assess the acute ventilatory effects of exposures in the workplace over the period of one work shift (so-called "shift" studies).

The Occupational Safety and Health Administration (OSHA) currently requires "shift" testing of cotton workers to identify those who experience an FEV_1 decrement of 5% or more,[48] commonly referred to as cotton dust "reactors." However, this degree of FEV_1 change is best used as a sensitive screening tool and needs repeat testing for confirmation.[37] In fact, 14% of a large population of unexposed workers had FEV_1 reductions at least this large over the course of their workshift.[49] A more common criterion categorizing FEV_1 shift-related declines of at least 10% over a workshift is largely empirically based, but may be justifiable for relatively healthy workers on the basis of intrasubject coefficients of variation for FEV_1 discussed earlier.[25] Furthermore, using the traditional statistical approach of defining an "abnormal" range as the lowest 5% of a gaussian distribution of "normal" values,[26] the 10% criterion fits well with "shift" study results from a large population of unexposed workers.[49]

A recent publication states that "there is no general agreement on what decrement of function over a work shift is necessary to make a diagnosis of occupational asthma."[50] Any chosen criteria for determining the "significance" of a decline are unlikely to be optimal for all purposes in all situations, and a theoretic discussion of a rational means for selection of a criterion to identify occupational asthma has been published.[51] In another vein, it is possible that with further evaluation of how best to express the difference between two FEV_1 tests on the same individual, use of absolute change rather than percentage change will prove superior, particularly for epidemiologic studies in which subjects are not as closely studied as they may be in the clinical setting.[52]

Although useful, the "shift" study approach has major limitations. Shift testing for epidemiologic studies is often done on only one day. Thus, particularly for industrial processes that are intermittent in nature, a representative exposure may not occur on the day of testing. Beyond that potential pitfall, many workers with occupational asthma may have a relatively fixed impairment of airway function from repeated expo-

short time course of responses to nonspecific challenges, responses can be delayed, prolonged, and difficult to reverse with bronchodilators.[56, 69] False-negative tests may result from inappropriate selection of the form of challenge agent, whereas false-positive data may result from challenge with unrealistically high doses that cause irritative bronchoconstriction. If considered only from an ethical perspective, use of specific challenges should remain largely restricted to the clinical evaluation of individual cases because exposures involve administration of potential sensitizers.

ENVIRONMENTAL ASSESSMENT

The environmental assessment serves a number of distinct purposes. It may be conducted to respond to specific complaints, to evaluate worker exposures to specific agents, to determine compliance with specific recognized workplace exposure standards, and to evaluate the effectiveness of engineering controls.[70]

Furthermore, the environmental assessment should assist in the design of environmental exposure control technologies. Occupational exposures can be controlled by application of engineering controls, proper design of work practices, and use of personal protective equipment. These measures may be applied at or near the source of exposure, at the actual point of exposure to worker(s), or within the general workplace environment. Engineering measures applied at the source of the hazardous exposure, including materials substitution, process/equipment modification, isolation or automation, and local exhaust ventilation, are generally preferred and provide the most effective means of control. Controls that may be applied to hazards that have escaped into the workplace environment include dilution ventilation, dust suppression, and housekeeping. Controls that may be applied to individual workers include the use of remote control rooms, isolation booths, fresh-air showers, work practices, and personal protective equipment. In general, a combination of all three types of control measures is required to provide worker protection. Process and workplace monitoring devices, personal exposure monitoring, and medical monitoring are important mechanisms for providing feedback concerning the effectiveness of the controls in use. Ongoing monitoring and maintenance of controls to ensure proper use and operating conditions, and the education and commitment of both workers and management to occupational health, are also important elements of an effective control program.[71]

The environmental assessment should be conducted by an experienced industrial hygienist. However, the physician should be knowledgeable of potential workplace hazards, and should be prepared to assist in identifying pertinent physical or chemical exposures that must be evaluated. In general, the environmental evaluation will require that the investigative team become familiar with all processes. Chemical and physical agents must be inventoried, job activities and work practices reviewed, sources of air contaminants and physical agents identified, and existing control measures studied (i.e., process enclosures and shielding, local and general exhaust ventilation, and imposition of barriers through the use of personal protective equipment).[72, 73] If personal protective equipment is used, then a program of training and maintenance in the use of equipment should be in place, and use of personal protective equipment, when necesssary, should be mandated by management and not optional on the part of the employee.

In regard to the conduct of the environmental survey, it is the industrial hygienist who must determine how, when, where, how long, and how many environmental samples should be obtained. It is particularly important to know if the device(s) and/or method(s) are specific for the contaminant(s) to be measured and what other substances interfere with the procedure(s). Issues of reliability and validity arise just as in the epidemiologic study. More than 200 chemical and physical agents have been implicated as causes of occupational asthma.[35] It is therefore neither practical nor feasible to recommend in this general discussion specific sampling strategies for all such workplace contaminants known to be associated with occupational asthma. It is appropriate to state that sampling should be conducted in accordance with accepted standard methodologies where available, and that the samples be analyzed in accordance with accepted standard analytic procedures.

Immunochemical assessment. It should be recognized that new, useful techniques evolve that do not gain immediate understanding or acceptance. Immunochemical assessment of exposures to biological substances in the workplace, such as animal- and plant-derived proteins, comprise a set of such procedures and will be briefly reviewed.

The application of immunochemical techniques for the detection and quantification of aeroallergens in the workplace holds promise as an effective means to enhance the environmental assessment. The basic concerns that need to be considered in using this methodology include sample collection, extraction, and analysis. In general, the collection and extraction methodologies are similar to what would be done for a variety of types of materials, but, because allergens can provoke a reaction at concentrations at which dust levels are low, some added precautions are necessary.

should not be selected merely because they have volunteered to participate in the study. Reasons for refusal to participate should be examined when possible.

E. Although personal characteristics such as atopy should be determined, the investigator must bear in mind that in a cross-sectional study, no conclusive inference can be drawn about personal characteristics and risk for developing disease.

F. Researchers should recognize that in an employee population, a disease with a high incidence may nonetheless have a low prevalence if mean duration of disease is short. The severely ill may no longer be present at the worksite. Therefore, efforts should be made to identify not just all current employees, but former employees as well. Attempts should be made to ascertain their reasons for leaving employment. Such followup of former workers may enable investigators to speculate whether prevalence measures derived from the initial study under- or over-represent the true magnitude of disease.

II. The questionnaire.

A. Experience does not currently warrant recommendation of a single "best" questionnaire for assessment of occupational asthma.

B. It is advisable to include questions from available, published questionnaires to ask about asthma symptoms because such questions have already been largely "debugged" in terms of ease of understanding and lack of ambiguity.

C. When choosing questions to assess work-relatedness of symptoms, it is best to allow for a variety of temporal patterns in relations to work (e.g., weeknight symptoms, Monday morning symptoms, etc.), especially if the suspect asthma-causing agent is a low molecular weight compound likely to cause late reactions.

D. The experience reviewed with the MRC and ATS-DLD questionnaires suggests that a single symptom like wheezing may not have the desired sensitivity. It is therefore preferable to use more than one symptom in combination with work relatedness to screen workers for further evaluation.

E. In order to assess the validity of a questionnaire for occupational asthma, a standard must be chosen that is independent of the questionnaire itself and is applied to both those with a positive and negative questionnaire screen. Symptoms and physiologic changes should be described separately, so that the relationship between outcome variables may be examined as well as the relationship of each outcome with the independent variable(s). "Clinical" diagnoses translated into case definitions tend to incorporate, to an unknown and/or unrecognized extent, the results of the tests under consideration and should be avoided.

F. Investigators who are not using previously published questionnaires should publish any questions used in their analysis.

G. Various combinations of symptoms used as criteria for a positive "screen" on the questionnaire should be assessed.

III. Pulmonary function testing.

A. Whenever feasible in the epidemiologic assessment of occupational asthma, objective pulmonary function testing on more than one occasion should be used to document variable airways obstruction that is temporally related to workplace exposure.

B. Questionnaire responses that describe the temporal pattern of asthma symptoms should be considered in deciding whether a single "shift study" spirometry survey will be likely to succeed in documenting most work-related airway responses, or whether longer term serial monitoring of peak flow should be done. If necessary, consideration should be given to making observations during and after a week or longer away from work.

C. Current widely used criteria for documenting variable airways obstruction include a 10% or greater reduction in FEV_1 over a single work shift or a 20% or greater reduction in peak flow over the course of a single day. Other criteria may be used, but should be justified with supporting statements.

D. When spirometry is used, American Thoracic Society recommendations for standardization should be followed. The investigator should limit unintentional bias by equivalent pulmonary function testing of both study and control subjects.

E. Airways "hyperreactivity" can be documented with nonspecific bronchial challenge testing, but should not be strictly

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