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Detection of microbial antigens in metal working fluids

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Abstract

In recent years there have been several reports of hypersensitivity pneumonitis (HP) or an HP-like illness occurring among machinists working with water-based metal working fluids (MWF). Microbial contamination of the MWF is common and microbial agents have been suspected to be causal agents for the HP-like illness, but no specific etiologic agent has been identified to date. In particular, gram negative bacteria and biocide resistant mycobacterial species may colonize the MWF, and may stimulate an inflammatory response if inhaled. Because direct culture techniques provide data only about viable organisms present at the time the sample was collected, we have been evaluating techniques to detect microbial substances (antigens) that may be present and persist in the MWF. We have found that the endotoxin of gram negative bacteria can be detected in MWF using the limulus amoebocyte lysate (LAL) assay, and may be present in high concentrations. In addition, MWF samples have been analyzed by Western Blot techniques using polyclonal antibodies to mycobacteria to demonstrate the presence of mycobacterial antigens in these samples. The use of non-culture-based techniques for the assessment of microbial contamination of MWF may help to determine the role of microorganisms in the etiology of HP associated with MWF exposure. Published by Elsevier Science Ltd.

Keywords: Metal working fluids; Microbial antigens; Endotoxin; Immunoassays

1. Introduction

Several recent reports have documented that occupational exposure to metal working fluid (MWF) mists is associated with respiratory symptoms (Sprince et al., 1997; Robins et al., 1997). Both occupational asthma (OA) (Rosenman et al., 1997) and hypersensitivity pneumonitis (HP) (Bernstein et al., 1995) have been linked with such exposures and these findings have increased concerns about the types of adverse pulmonary reactions that may affect machinists. In 1997 the National Institute for Occupational Safety and Health (NIOSH) at the request of the United Auto Workers (UAW) convened a workshop to review the available data from eight outbreaks of HP that had occurred in the automotive industry (Kreiss and Cox-Ganser, 1997). Although no single etiologic agent was identified, a common finding was that the clusters of HP were associated with the use of water-based MWF. Rosenman et al. (1997) reported an association between the occurrence of work-related asthma and respiratory symptoms among machinists and the use of water-based MWF as well. The water-based MWF may become contaminated with bacteria, and thus the potential role of microbial agents in these outbreaks is of interest.

Machining fluids can be divided into four general categories: straight, soluble, semi-synthetic, and synthetic oils. The soluble, semi-synthetic and synthetic oils are water-based MWF and in use are emulsions that contain up to 95% water by volume. Other chemicals may be added to the MWF to enhance the performance of the fluid or to improve the useful life of fluid. Among the chemical additions to the water-based MWF are biocides that act to prevent or control microbial growth in these fluids as microbial contamination may have a severe adverse effect on the performance of the fluids. Indeed, most of the research on the microbiology of MWF has been focused on the degradation of the fluids by microorganisms, and methods to control microbial growth.

The association of respiratory complaints, in general, and HP, in particular, to exposure to water-based MWF raises concerns that these diseases may be due to the inhalation of specific microbial agents or substances. Microorganisms produce a variety of substances that can stimulate inflammatory, toxic, or allergic (immune) reactions when inhaled. Numerous studies have found that gram-negative bacteria especially *Pseudomonas* spp. are the major bacterial

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contaminants of MWF (Rossmoore, 1986). Antibodies to *Pseudomonas* spp. have been found in the sera of subjects with MWF-associated HP (Bernstein et al., 1995). The endotoxin derived from gram-negative bacteria are well studied, potent inducer of inflammation (Olenchock, 1994), and inhalation of endotoxin containing MWF mists may contribute to the pulmonary response. In addition, the use of chemical sterilants (biocides) to control microbial growth in MWF may select for the growth of resistant species, especially the atypical mycobacteria. The cell wall components of mycobacteria such as muramyl dipeptide, trehalose dimycolate and glycopeptidolipids are potent immune system adjuvants (Gjata et al., 1994) and mycobacterial extracts are often mixed with other antigens to enhance the antibody responses of experimental animals. Atypical mycobacteria have been isolated from some but not all of the MWF samples associated with outbreaks of HP. Thus, these two groups of microorganisms are considered important in the etiology of HP as potential sources of antigenic material, as inducers of inflammation, or as immunologic adjuvants.

Our laboratory has participated in the investigation of several outbreaks of HP associated with exposure to MWF. Rather than present the results of those studies, several of which are continuing, this report will focus on the methods and techniques we have adopted to study the microbial substances that may be present in MWF. In particular, the development of non-culture based techniques to detect and quantify microbial substances in used MWF samples will be described. This approach to the study of microbial agents in MWF evolved from the recognition that the adverse pulmonary reactions associated with MWF exposure are not due to viable organisms per se but rather to inflammatory or immune reactions to microbial products. In addition, microbial culturing techniques may significantly underestimate the total microbial biomass due to the use of either selective media, the presence of non-culturable organisms, or the presence of rapidly growing organisms that mask the detection of more slowly growing organisms such as the mycobacteria. To address these concerns we have developed or adapted sensitive assays for specific microbial products or antigens for use with MWF samples. Also, the monitoring of sera from exposed workers for specific antibodies to microbial contaminants can be used as a biomarker of exposure, and we have adapted these techniques for use with MWF samples. Antibody positive sera have in turn been used to analyze MWF samples by immuno-blotting techniques. Such assays can provide information about the antigenic composition of MWF samples, or confirm the presence of a specific antigen or agent in the samples.

2. Methods

2.1. Endotoxin analysis

Bulk samples of MWF were assayed for endotoxin content using a limulus amebocyte lysate (LAL) assay (Kinetic-QCL, BioWhittaker Inc., Walkerville, MD) according to the kit manufacturer's recommended procedures. Briefly, the MWF samples were allowed to warm to room temperature, and vigorously mixed (vortexed) for 5 min. Two 3 ml samples were removed from the bulk fluids and a series of five-fold dilutions were prepared from each. The samples were vortexed for 3 min before being diluted and again before being dispensed in the assay plate. The concentration of endotoxin was determined by reference to a standard curve prepared for each assay and which had a linear range from 0.005 to 50 Endotoxin Units/ml (EU/ml). Airborne endotoxin levels were determined from glass fiber filters used to collect air samples which were stored at 4°C in their cassettes until extraction. The filters were aseptically transferred to sterile 50 ml centrifuge tubes, and extracted with 10 ml of pyrogen free water for 60 min at room temperature on a platform rocker. The samples were then centrifuged for 10 min at 2500 rpm, and the supernatant fluid was recovered and stored at −85°C until assayed for endotoxin content.

2.2. Preparation of antigens

Isolates of *Mycobacterium chelonae* (*M. chelonae*) were obtained from a commercial microbiology laboratory and had originally been isolated from and identified as the predominant microbial contaminant in selected MWF bulk samples. Isolates were grown in R2A broth at 30°C with constant stirring in a rotary incubator set at 80 rpm for 4–6 weeks. The purity of the cultures were confirmed by acid-fast staining at the beginning and end of the incubation period. The bacterial cells were recovered by centrifugation (2500 rpm/15 min), washed twice with sterile saline, and resuspended in saline as a 10% (vol/vol) suspension. The bacterial suspensions were sonicated for one minute using a Branson Model 350 sonifier set at 40% output, 50% duty cycle pulse. The sonicates were clarified by centrifugation at 3500 rpm for 20 min, and the supernatant fluid filtered using a 0.45μ polycarbonate filter. The protein content of bacterial sonicate was determined by a modified Lowry (Bio-Rad Laboratories, Inc. Hercules, CA) according to the manufacturer's recommendations, and the samples stored at −20°C.

In order to investigate the antigenic composition of the MWF samples it was necessary to first break the emulsion so that the aqueous phase could be recovered. This was accomplished by subjecting the samples to centrifugation at 10,000 g for 3 h at 4°C. The aqueous phase was recovered, dialyzed against carbonate coupling buffer (pH 9.3), and stored at −20°C until used. The protein content was determined by the Lowry method, and the samples diluted to contain approximately 5 µg protein/ml for use in the ELISA.

2.3. ELISA procedure

A direct enzyme linked immunosorbent assay (ELISA) for antibodies to *M. chelonae* or MWF extract was

developed using procedures described by Carpenter (1992). Briefly, ELISA plates were coated with the *M. chelonae* or MWF extract (3–5 μg protein/ml) in carbonate coating buffer overnight at 4°C, blocked with 1% human serum albumin, and stored at 4°C until used. The subject sera were initially tested in duplicate at a 1 : 80 dilution, and antibody binding was detected using peroxidase labeled anti-human immunoglobulins and developed with a tetramethyl benezidine (TMB) substrate (Sigma Immunochemicals, St. Louis, MO). Appropriate positive and negative controls performed with each plate included a blank well (no antigen) to correct for non-specific binding of secondary antibody, an antigen control developed with rabbit anti-mycobacteria, a negative control using serum from an individual with no known exposure, and a positive control using serum from an individual with antibodies. A series of preliminary assays were conducted to identify potential positive and negative controls for each study. To verify that the positive control serum contained antibodies, inhibition studies using a soluble form of the target antigen were performed. Dilutions of the test sera were incubated with soluble antigen extract for 1 h and then assayed as usual. Dose-dependent inhibition of immunoglobulin binding by soluble antigen indicated that antibody was being detected by the ELISA. Inhibition studies with sera showing low binding levels in the ELISA yielded absorbency values similar to the antigen control wells and were used to defined non-specific binding levels. Results were reported in terms of absorbency units, or as antibody positive and negative. An ELISA positive reaction was defined as an absorbency value greater than the mean plus two standard deviations of 32 inhibition assays.

2.4. *Immuno-blotting techniques*

To detect antigenic proteins in the MWF samples, the samples were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). The MWF samples were vigorously mixed and 50 μl added to a equal volume of SDS–PAGE buffer containing dithiothreitol (3% w/v) and the mixture boiled for 5 min before being loaded onto 12% gels. The separated proteins were transferred to nitrocellulose membranes using a tank transfer system (BioRad). The membranes were washed using a 1% dried milk solution to block unreacted sites and reacted with antisera or antibody positive serums. Mycobacterial antigens were detected using rabbit anti-mycobacteria (BioDesign International, Kennebunk, ME) and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma Immunochemicals, St. Louis, MO) and human antibodies were detected using HRP conjugated goat anti-human IgG, Fc specific (Sigma) and the HRP binding visualized using TMB as a substrate. Optimal dilution for each reagent was determined by "dot-blotting" techniques prior to the immuno-blotting studies. For inhibition studies the sera were incubated with soluble antigen extract for 1 h at room temperature before the sera was diluted and reacted with the membranes.

3. Results and discussion

Endotoxin analysis of bulk MWF samples revealed that these fluids may contain relatively high levels of endotoxin. As shown in Table 1, the endotoxin content in MWF from three separate studies varied greatly; ranging from undetectable to over a million EU/ml of samples. The components of the MWF can interfere with the LAL assay, but by dilution it is usually possible to obtain valid measurement. This type of interference would mean that samples with lower levels of endotoxin may appear to have undetectable levels. Because the interfering substance(s) will vary qualitatively and quantitatively between types of MWF and may even change with use, the lower limits of detection for the LAL assay with these samples cannot be defined. The data do show that on occasion the endotoxin levels can be quite high. In the few cases where we have both bacterial culture results and endotoxin data, there is good agreement between endotoxin levels and the gram negative bacteria counts (data not shown).

The presence of antibodies in the sera of exposed individuals can be used as a marker of exposure. With respect to MWF samples, the antibody data can be used to provide evidence that dermal or inhalation exposure to the MWF lead to an immune response to a contaminant in the fluid. The resulting antibodies can be used to help identify if a particular agent is present in the fluid samples. ELISA's to both bacterial isolates and MWF extracts can be developed and as shown in Table 2 antibodies to both a specific bacterial isolate and the MWF extract can be found in the sera

Table 1
Endotoxin content of metal working fluid samples

	Study 1	Study 2	Study 3
Number of samples	76	11	41
Samples containing endotoxin (%)	55 (72%)	11 (100%)	22 (54%)
Mean ± S.D. (EU/ml)	68,600 ± 172,000	97,000 ± 109,000	59,200 ± 104,400
Range (EU/ml)	nd–1,011,000	90–289,000	nd–427,000
Aerosol (EU/mg) $N = 35$	35.7 ± 26.3		
Aerosol (EU/M^3) $N = 35$	1.5 ± 1.0		

Table 2
Results of ELISA assays: Prevalence of antibodies to *M. chelonae* or MWF extracts

Exposure status	*M. Chelonae* (%)	MWF (%)
Exposed ($n = 177$)	35	39
Non-exposed ($n = 60$)	25	10
Total ($n = 237$)	32	32

of exposed workers. In this case "exposed" implies machinists and "non-exposed" employees worked in other area of the same plant and the assignment of an individual into a particular group was based on questionnaire results. Subjects in the non-exposed group had to have not worked in the machining area for at least six months prior to study. The data demonstrated that the prevalence of antibodies for an environmental organism like *M. chelonae* was fairly high in the non-exposed population suggesting that one needs to be cautious interpreting the data. Mis-classification of exposure status is possible and exposure may occur outside the work environment. The data in this table also shows that using the MWF extract, the antibody data more closely matched the exposure status as defined by job description. While not defining a specific agent, this type of data may identify workers who have more intimate contact with the fluid. It is possible to obtain antibodies to many common microorganisms from commercial sources, and these commercial reagents can be used to validate the ELISA; however, when environmental extracts are used validation of the ELISA may be more difficult. Sera from individuals with no known exposure can be used as negative controls, but positive controls are lacking. For this study we evaluated the sera of a number of exposed workers, identify some that have reactivity to the extract, and use those "positive" sera to optimize the ELISA procedure. Optimization of an ELISA procedure involves determining a screening dilution at which antibody negative sera and non-specific binding controls (i.e., no antigen present) give similar values, and then evaluating time and temperature of the incubation steps to obtain maximal values from the antibody positive sera. Inhibition studies were conducted to confirm antibody activity.

Another technique useful in evaluating the immune response to microbial contaminants in MWF is immuno-blotting procedures, also known as Western Blotting. Working with sera that were identified as having antibodies to a MWF extract by the ELISA procedure, we found that the SDS–PAGE procedure apparently removed the interfering substances present in MWF samples so that protein antigens present in the samples could be detected without breaking the emulsions. Results of such an analysis are shown in Fig. 1. In this experiment the sera from several antibody positive individuals were combined and used to probe the antigenic composition of different MWF samples. Inspection of Fig. 1 shows that the samples differed greatly both quantitatively and qualitatively in antigenic composition even though these were similar types of fluid from the same facility. The immuno-blotting technique was the only method that revealed the protein content of these samples. We were unable to determine the protein content of these samples by colorimetric methods such as the Lowry procedure, and direct staining of the gels for protein with either Commassi blue or silver staining was not successful. The quality and specificity of the antisera used to probe the membrane is of critical importance in doing this type of analysis. The lack of staining does not mean that antigenic material is absent. The specific antigen recognized by that antisera may be absent or too dilute to be detected, but other antigens may be present.

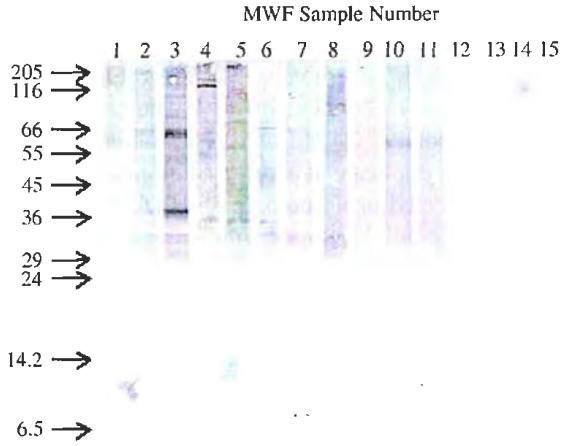

Fig. 1. Immunoblot of 15 MWF samples. Number across the top refer to the sample number and the number with arrows, the position of molecular weight markers (in kDa). The immunoblot was developed using a pool of sera from individuals who had antibodies to *M. chelonae* as determined by an ELISA assay.

When one or a limited number of MWF samples are thought to be the source of antigenic material, it is possible to adapt the immuno-blotting procedure so that a number of serum samples can be assayed. It is possible to prepare the SDS–PAGE gels that have only one well that covers most of the width of the gel and load the gel with one sample. After transfer, the membrane can be placed in a chamber that has a number of thin grooves so that several serum samples can be tested at the same time. Fig. 2 is an example of such an analysis. This approach allows for the assessment of several serum samples at the same time, but the results should be considered qualitative; that is, only the presence or absence of antibodies to a protein of a particular molecular size may be determined. A useful variation of this procedure for the identification of microbial antigens is an inhibition study. For this type of analysis, an aliquot of the sera is reacted with an extract of the suspect microorganism and banding pattern with and without inhibitor present provides evidence that an antigen was from that organism. We have done such studies to show that binding to a 29 kDa protein in the MWF samples could be inhibited by preincubation with a *M. chelonae* extract, but that binding to other antigens was not affected. These data would indicate that a species of Mycobacterium was present in the fluid, but was not the sole source of antigenic material. It may be that the well-known adjuvant activity of mycobacterial cell wall components may have promoted the antibody response to other antigenic substances present in the fluid.

Antisera specific for suspected organisms can be used in immuno-blotting protocols to detect those microbial antigens. We used a rabbit antisera to mycobacterial antigens to

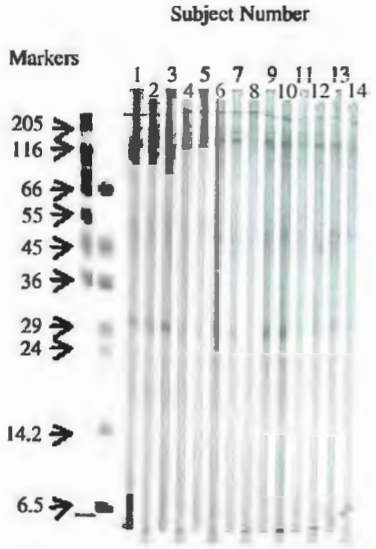

Fig. 2. Immunoblot of a MWF sample probed with multiple serum samples. The MWF sample #3 in Fig. 1 was subjected to SDS–PAGE separation and reacted with 14 *M. chelonae* ELISA positive sera. Subject #8 showed no reactivity with the sample, while the remaining sera showed the major antigens had molecular sizes of approximately 120 and 29 kDa, and other antigens present as well.

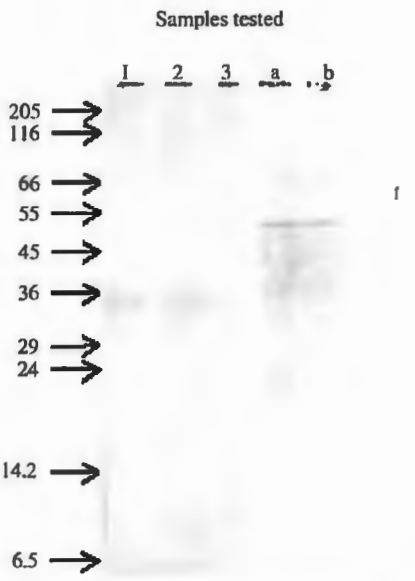

Fig. 3. Immunoblot of MWF samples developed with rabbit antisera to *M. chelonae*. Lanes labeled 1, 2 and 3 were MWF samples suspected of containing *M. chelonae*, and lanes labeled "a" and "b" were the *M. chelonae* sonicate tested undiluted (a) and diluted 1 : 2 (b). Rabbit antisera to *M. chelonae* was used to develop the immunoblots.

probe for the presence of the antigens in MWF samples. As shown in Fig. 3, two of three MWF samples assayed with anti-mycobacterial serum showed a 35 kDa antigen present. For comparison, an immuno-blot of a *M. chelonae* extract react with the anti-mycobacteria rabbit serum is shown. A major band at approximately 53 kDa is apparent, and a diffuse "smear" of antigenic material in the 20–50 kDa range can be seen. These results show that not all of the *M. chelonae* antigens recognized by the rabbit antisera are present in the MWF samples, but at least one is. This may represent either degradation of the mycobacterial antigens in the MWF or the sensitivity of the assay.

In summary, the analysis of MWF samples for the presence of microbial substances that contribute to occupational respiratory diseases present a challenge to immunologists and microbiologists. Traditional microbial culturing techniques may not provide reliable or relevant data in that the pulmonary response is more likely due to an allergic or inflammatory reaction that is not dependent on exposure to viable organisms. The analysis of endotoxins from gram negative bacteria by the LAL assay is a method that can be adapted for use with the MWF samples in part because this is such a sensitive assay that interfering substances present in the MWF can be diluted. Detecting antibodies to antigenic substance in the MWF requires that the emulsions be broken and this can be difficult. When the emulsions can be successfully broken, then ELISA assays can be developed that will allow for the screening of a large number of serum samples. When the emulsions cannot be easily broken, the use of immuno-blotting techniques allows for the analysis of antigenic composition of the MWF samples. These techniques can be adapted to screen multiple MWF samples for the presence of common antigens, or to screen multiple serum samples for antibodies to a particular MWF sample. Such information can be used in support of clinical and environmental studies and may help identify exposure sources or possible etiologic agents in outbreaks of respiratory diseases among machinists.

References

Bernstein, D.I., Lummus, Z.L., Santilli, G., Siskosky, J., Bernstein, I.L., 1995. A hypersensitivity pneumonitis disorder associated with exposure to metal-working fluids aerosols. Chest 108, 636–641.

Carpenter, A.B., 1992. Enzyme-Linked Immunoassays In: Rose, N.R., de Macario, E.C., Fahey, J.L., Friedman, H., Penn, G.M. (Eds.), Manual of Clinical Immunology. Fourth Edition. American Society for Microbiology, Washington, D.C., pp 2–9.

Gjata, B., Hannoun, C., Boulouis, H.-J., Neway, T., Pilet, C., 1994. Adjuvant activity of polar glycopeptidolipids of *Mycobacterium chelonae* (pGPL-Mc) on the immunogenic and protective effects of an inactivated influenza vaccine. C.R. Acad. Sci. Paris/Life Sciences 317, 257–263.

Kreiss, K., Cox-Ganser, J., 1997. Metalworking fluid-associated hypersensitivity pneumonitis: a workshop summary. American Journal of Industrial Medicine 32, 423–432.

Laemmli, U.K., 1970. Cleavage of structural protein during the assemblage of the head of bacteriophage T4. Nature (London) 227, 680–685.

Olenchock, S.A., 1994. Health effects of biological agents: the role of endotoxins. Applied Occupational Environmental Hygine 9, 62–64.

Robins, T., Seixas, N., Franzblau, A., Abrams, L., Minick, S., Burge, H., Schork, M.A., 1997. Acute respiratory effects on workers exposed to metalworking fluid aerosols in an automotive transmission plant. American Journal of Industrial Medicine 31, 510–524.

Rosenman, K.D., Reilly, M.J., Kalinowski, D., 1997. Work-related asthma and respiratory symptoms among workers exposed to metal-working fluids. American Journal of Industrial Medicine 32, 325–331.

Rossmoore, H.W., 1986. Microbial degradation of water-based metalworking fluids. In: Moo-Young, M., Cooney, C.L., Humphres, A.E. (Eds.), Comprehensive Biotechnology. Pergamon Press, New York, NY, pp. 249–269.

Sprince, N.L., Thorne, P.S., Popendorf, W., Zwerling, C., Miller, E.R., DeKoster, J.A., 1997. Respiratory symptoms and lung function abnormalities among machine operators in automobile productions. American Journal of Industrial Medicine 31, 403–413.