The Antibody Response to Methyl Isocyanate: Experimental and Clinical Findings

by Meryl H. Karol,∗ Saroj Taskar,† Sudha Gangal,‡ Beverly F. Rubanoff,* and S. R. Kamat†

As a result of the industrial accident in Bhopal, India (December 1984) in which thousands of people were exposed to methyl isocyanate (MIC), concern was raised for possible long-term health effects. The well-recognized immuneological consequences of exposure to other industrial isocyanates prompted investigation of an antibody response to MIC. Using procedures which had been developed in this laboratory to evaluate isocyanate immunotoxicity, animal studies were undertaken to develop and test reagents which could be used to detect antibodies to MIC in the exposed population. Guinea pigs were injected with MIC in its reactive isocyanate form. Three weeks later, blood was drawn and serum evaluated using ELISA. To detect antibodies, an antigen was prepared by reaction of MIC with guinea pig serum albumin. Antibodies were detected in each of the four animals injected with MIC. Titers achieved were 1:5120 to 1:10,240. Inhibition assays revealed antibody specificity directed toward the MIC hapten. Analogous antigens prepared by reaction of MIC with human serum albumin were used to evaluate sera from individuals exposed in Bhopal to MIC. Antibodies were detected in 12 of 144 exposed persons. Antibodies were specific for MIC, as evidenced by inhibition assays, and belonged to the IgG, IgM and IgE classes. However, titers were generally low and transient and were found in persons having had the highest MIC exposures. Total IgE values of sera were not significantly different from those of control sera obtained from Bombay residents. The results indicate that exposure to methyl isocyanate resulted in production of specific antibodies. However, the low titers observed and the transient nature of the response suggest little health consequence should result from the antibody response.

Introduction

The accident on the night of December 3, 1984, in Bhopal, India, resulted in the exposure of thousands of residents to methyl isocyanate. At the time, little was known about either the long- or short-term effects of exposure to this chemical. However, a considerable body of information existed regarding the health effects of other industrial isocyanates, most notably toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI), and hexamethylene diisocyanate (HDI) (1,2).

Isocyanates are recognized as chemicals capable of causing immunologic sensitization (3). Respiratory hypersensitivity, including immediate and delayed onset pulmonary reactions (4,5), as well as hypersensitivity pneumonitis (6,7), has been noted in individuals exposed to certain isocyanates. Dermal sensitization has been reported following exposure to other isocyanates, notably to dicyclohexylmethane diisocyanate (HMDI) (8,9).

Respiratory sensitivity and dermal sensitization have been reproduced in animals by the use of inhalation (10–13) and/or dermal (14) routes of exposure. The literature regarding isocyanate sensitization has been summarized and critically evaluated (1–3).

Recent concern has addressed exposure conditions which would favor sensitization (1,2). Both animal data (12,15) and human studies (16–18) indicate a concentration-dependence of the specific humoral immune response to toluene diisocyanate and dose-dependent dermal sensitization to TDI, HDI, MDI and HMDI (19). Due to the causative association of IgE antibodies with immediate, and possibly late-onset pulmonary hypersensitivity reactions (20,21), interest has focused on the detection of this class of antibody following accidental isocyanate exposure (1,17).

The massive MIC exposure in India prompted immediate investigation of the immune response to this isocyanate. The study reported here identified a humoral immune response to MIC, as tested in an animal model for isocyanate sensitization. Development and testing of appropriate immunologic reagents in the an-
imal system allowed detection of low levels of MIC antibodies in persons who had received heavy MIC exposure.

Materials and Methods

Animals

Male, English, smooth-haired guinea pigs weighing approximately 500 g (Hilltop Lab Animals, Inc., Scottsdale, PA) were used for production of antibodies.

Antigens

Isocyanate conjugate antigens were prepared according to a standard procedure (22). Basically, the isocyanate was added dropwise to a stirred solution of protein dissolved in 0.05 M borate-KCl buffer, pH 9.4, maintained in an ice bath. A molar ratio of 100:1, isocyanate:protein was typically employed. Reactions were terminated by addition of excess monoethanolamine (Fisher Scientific). Conjugates were purified by isoelectric precipitation followed by dialysis in 0.1 M sodium bicarbonate solution, dialysis, then lyophilization. Yield was usually greater than 90%. The reaction time and temperature were varied to attain substitution of approximately 20 to 30 mole hapten/mole protein. The following antigens were prepared: MIC-guinea pig serum albumin (MIC-GSA); MIC-human serum albumin (MIC-HSA); MIC-keyhole limpet hemocyanin (MIC-KLH); TDI-GSA, TDI-HSA, hexamethylene diisocyanate-GSA (HDI-GSA).

Production of Antibody to MIC

Four guinea pigs received intradermal injection with 50 μL of 4% MIC (in acetone) into each of two shaved sites in the shoulder area. Twenty-four days later they received a second set of injections with 2% MIC. Blood was drawn prior to MIC exposure and again on day 31.

Production of Antibody to MIC-HSA

Four animals were injected with MIC-HSA to provide positive control sera to detect human antibodies reactive with MIC. For this purpose, animals were injected SC with an emulsion containing equal volumes of a 0.5% solution MIC-HSA (in saline) and Freund’s complete adjuvant (FCA, Difeo, Detroit, MI). Two sites each received 200 μL. On days 3 and 21, animals received booster injections each containing 1.5 mg MIC-HSA in incomplete Freund’s adjuvant. Blood was drawn on day 28.

Antibody Assays

ELISA (Enzyme-Linked Immunosorbent Assay). ELISA was performed to evaluate both human and animal antisera for the presence of antibodies to methyl isocyanate. Ninety-six well polystyrene plates were coated with 100 μL antigen (5 μg/mL in 0.1 M carbonate buffer, pH 9.6). Antisera were added in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), containing 0.5% Tween 20 (Fisher Scientific Co.) and 0.5% BSA (Sigma Chemical). The final volume in each well was 100 μL. Plates were incubated for 1 hr at 37°C, washed three times with tween-saline, then reacted with peroxidase-conjugated anti-guinea pig (or anti-human) reagent (1:400 dilution). The following reagents were used: antiguinea pig IgG, anti-human IgG, IgM, and IgA (Miles-Yeda). After incubation at 37°C for 1 hr, plates were washed, then a solution containing 0.72 mg/mL 5-aminosalicylic acid (Aldrich Chemicals) and 0.006% H2O2 was added, and incubation continued at 37°C for 30 min. Absorbance at 450 nm was measured using a Dynatech MR 600 Microplate Reader. The titer was defined as the highest serum dilution giving an absorbance at least twice that of control sera, similarly diluted, after subtraction of background.

ELISA inhibition assays were performed by incorporating antigen as inhibitors into the tween-PBS buffer immediately prior to dispensing antisera into the wells. IgE. The total quantity of serum IgE was determined using a commercial IgE kit (Ventre-Sep, Ventrax).

RAST (Radioallergosorbent Test). RAST was performed by coupling MIC-HSA to cyanogen-bromide activated paper discs using a standard procedure (17). The MIC-HSA antigens used had between 20 to 40 mole MIC bound per mole protein. For assay, 50 μL serum was incubated with a washed disc at ambient temperature for 16 hr. After thorough washing of the disc, 125I-labeled anti-human IgE (Ventrex Reagent) was added to detect bound IgE antibody. Titers were expressed as the percent of added radioactivity which remained bound to the discs following the final washing procedure. RAST inhibition was performed by reacting 25 μL serum with 25 μL inhibitor solution (containing 50 μg inhibitor) for 2 hr at 37°C prior to addition of the MIC-HSA disc. Thereafter, the RAST procedure was followed. Inhibitors included MIC-HSA and HSA. Percent inhibition was calculated as follows:

\[
\left(1 - \frac{\% \text{ CPM bound above blank with inhibitor}}{\% \text{ CPM bound above blank with buffer}}\right) \times 100
\]

Statistics

Linear regression analysis was performed using MINITAB. The two-tailed Student’s t-test was used to compare IgE values in control and exposed cases.

Results

Identification of MIC-Specific Antibodies in Animals

An animal model was used to determine the ability of MIC to function as a hapten following in vivo expo-
sure. Blood was drawn from MIC-exposed animals and evaluated for specific antibodies using ELISA. Each of the four guinea pigs injected with MIC produced antibodies. Titers are given in Table 1. The specificity of these antibodies for MIC was evaluated using ELISA inhibition assays. Inhibitors tested included various conjugates containing MIC hapten (MIC-GSA, MIC-HSA, MIC-KLH) to enable identification of hapten specificity of antibody, conjugates containing heterologous isocyanates (HDI, TDI) to indicate possible isocyanate reactivity, protein carriers (GSA, HSA) to indicate protein recognition, and a formaldehyde-GSA conjugate (23) to provide information regarding the fine specificity of antibody toward a small hapten. Inhibition assays were performed in triplicate. Results of these assays using sera from two different animals are shown in Figures 1A and 1B.

The specificity of antibodies for MIC was apparent. MIC-GSA, MIC-HSA, and MIC-KLH were effective inhibitors, whereas the unconjugated proteins, GSA, HSA, and KLH gave insignificant reaction with antibody even at the highest concentration tested (50 μg). The relative potencies of MIC-conjugates for reaction with MIC antibody are listed in Table 2; the statistical parameters of the regression lines are listed in Table 3. With both antisera, MIC-KLH was the most effective inhibitor, followed by MIC-GSA and MIC-HSA. The lack of inhibition shown by quantities up to 50 μg of TDI-GSA and HDI-GSA indicates that antibodies were not directed toward isocyanate groups on the conjugates. Failure of HCHO-GSA to effect inhibition further indicated the specificity of antibodies for MIC hapten.

### Specificity of Inhibition by MIC-KLH

Several reasons may be proposed for the greater ability of MIC-KLH, as compared with MIC-GSA and MIC-HSA, to inhibit reaction of the animal antisera. The possibility was explored that inhibition by MIC-KLH was not specific for MIC determinants, but rather was due to non-specific interference of MIC-KLH with the ELISA system. To test this possibility, MIC-KLH was assayed for inhibitory activity in a heterologous antigen-antibody ELISA system.

An ELISA assay had been developed in this laboratory to detect antibodies to bacterial subtilisin in guinea pigs exposed to the enzyme via the inhalation route (Hillebrand et al., personal communication). Using polyvinyl chloride 96 well microtiter plates (Flow Laboratories) coated with 50 μL of a 50 μg/mL subtilisin solution, the antiserum titer was 1:20,480. MIC-KLH in amounts of 0.1 and 1.0 μg was incorporated into the incubation buffer before addition of antiserum to the plate; GSA was incorporated into the buffer dispensed into control wells. Anti-serum to subtilisin was added. After 2 hr at 37°C, bound antibody was assayed by addition of alkaline-phosphatase conjugated antiguenine pig IgG followed by phosphatase substrate. The results of this assay are shown in Table 4. MIC-KLH gave no inhibition at 0.1 or 1 μg. These results indicated that the inhibition afforded by MIC-KLH in the MIC anti-

### Table 1. Antibody titers to MIC in guinea pigs.*

| Animal no. | Antibody titer |
|------------|----------------|
| 3060       | 1:10,240       |
| 3061       | 1:5,120        |
| 3062       | 1:10,240       |
| 3063       | 1:10,240       |

*Antibody titers were determined by ELISA using peroxidase-labeled anti-guinea pig IgG (1:400 dilution). Serum from the same animals taken prior to exposure showed no antibody titer.
Table 2. ELISA inhibition assays to indicate specificity of MIC antibodies raised in guinea pigs.

| Inhibitor  | µg Required for 50% inhibition* |
|-----------|--------------------------------|
| Serum #3060 | Serum #3063 |
| MIC-KLH    | 0.64       | 0.27       |
| MIC-GSA    | 2.90       | 2.89       |
| MIC-HSA    | 4.94       | 5.98       |
| TDI-GSA    | >50        | >50        |
| HDI-GSA    | >50        | >50        |
| HCHO-GSA   | >50        | >50        |
| KLH        | >50        | >50        |
| GSA        | >50        | >50        |
| HSA        | >50        | >50        |

*Inhibitors were incorporated into the assay buffer and dispensed into microtiter wells prior to addition of antibody. The amount required to effect 50% inhibition of reaction was obtained from linear regression analysis of dose-response data shown in Fig. 1.

Table 3. Regression parameters for inhibition regression lines in Figure 1.*

| Inhibitor | Correlation coefficient | Log slope |
|----------|-------------------------|-----------|
| Serum #3060 | Mic-GSA | 0.973 | 1.54 |
| Mic-HSA | 0.998 | 1.40 |
| Mic-KLH | 1.000 | 1.75 |
| Serum #3063 | Mic-GSA | 0.986 | 1.42 |
| Mic-HSA | 0.998 | 1.28 |
| Mic-KLH | 0.999 | 1.12 |

*Linear least-square regression analysis was performed using MINITAB.

Table 4. Test for inhibition by MIC-KLH in a heterologous antigen-antibody (subtilisin) system.*

| Antigen     | Absorbance at 410 nm |
|-------------|----------------------|
| None        | 0.930*               |
| Mic-KLH (0.1 µg) | 0.854       |
| Mic-KLH (1.0 µg) | 0.898       |

*MIC-KLH was added to microtiter wells that had been coated with subtilisin. Antisera to subtilisin was added and bound antibody detected using phosphatase-conjugated anti-guinea pig IgG.

*Each value represents the mean of triplicate determinations.

body system was specific and due to recognition of MIC antibodies by this antigen.

Evaluation of Sera from Bhopal Patients

Serum was collected from individuals exposed to methyl isocyanate at various times following the accident and evaluated for its IgE content (Ventex/Sep, Ventrex). These values are given in Table 5. In the exposed patients, there was no significant trend in these values throughout the year following exposure. Furthermore, in comparing sera from exposed persons with those from controls, no significant differences were noted, except in sera drawn in April 1985. However, no significance is attached to this finding because of the single sample constituting the control group.

RAST

RAST was performed to detect the presence of MIC-specific IgE antibodies. To determine a positive RAST titer, cellulose discs were coated with MIC-HSA, and the assay was performed with sera from control, non-exposed individuals from India. Values for RAST binding were regressed against values of total IgE in the sera. The regression is shown in Figure 2. A strong correlation existed between these assays; the correlation coefficient for the 41 sera was 0.9223. Using the 95% confidence limits for the line, RAST values above this limit were considered presumptively positive. Sera with such values were further evaluated using RAST inhibition for definitive identification of specific antibody. Inhibition of 25% or more by MIC-HSA at 5 µg was taken as evidence for the presence of MIC-specific antibody.

Results of RAST testing are found in Table 6. Five of the 128 patients tested had antibodies specific for MIC-HSA, with titers ranging from 6.3 to 11.4% binding. In several cases, additional serum samples collected at later time periods were available. Analysis of these samples indicated that the antibody titer typically decreased with time from exposure (Table 7).

IgG and IgM Antibodies to MIC

ELISA was used to evaluate sera for the presence of IgG, IgA, and IgM antibodies to MIC. Controls for these assays consisted of (a) sera from nonexposed Indian donors and (b) sera from guinea pigs which had been immunized with MIC-HSA (in Freund's adjuvant) and contained anti-MIC-HSA antibody titers in excess of 1:10,000. Antibodies were identified in 10 individuals. As indicated in Table 6, some patients formed antibodies of more than one class; usually IgG and IgM occurred together. Titers were uniformly low; a maximum titer of 1:128 was observed. In some cases, amounts of sera were insufficient to determine a final titer. However, in all cases, antibody specificity was confirmed by the ability of MIC-HSA to inhibit binding.

Relationship Between Exposure Concentration and Antibody Production

It was of interest to determine if a relationship could be discerned between the severity of exposure to MIC and development of anti-MIC antibodies. For this purpose, the actual exposure received by individual patients was estimated from evaluation of the symptoms exhibited at the time of exposure. These symptoms have been detailed elsewhere (24). In brief: exposure severity was assessed based upon physical examination, chest radiograph, spirometry, the patient's need for hospitalization, the duration of a serious condition, and occurrence of complications.

The development of MIC antibody in patients grouped according to exposure severity is given in Table 8. It is apparent from this table that IgG and IgM an-
**Table 5. IgE values in serum from individuals exposed to methyl isocyanate.*"**

| Date serum collected | Individuals exposed to MIC | Control individuals |
|----------------------|----------------------------|---------------------|
|                      | N  | IU/mL (SD)b       | N  | IU/mL (SD) |
| December 1984        | 13 | 362 (1018)        | 2  | 1247 (1923) |
| January 1985         | 107| 391 (1512)        | 13 | 1344 (1937) |
| February 1985        | 6  | 1104 (4755)       | 6  | 2288 (1998) |
| March 1985           | 49 | 398 (1415)        | 6  | 2288 (1998) |
| April 1985           | 25 | 286 (874)         | 1  | 4400        |
| May 1985             | 3  | 276 (693)         | —  | —           |
| June 1985            | 56 | 682 (2107)        | 12 | 424 (1198)  |
| July 1985            | 10 | 534 (1384)        | —  | —           |
| December 1985        | 22 | 441 (1967)        | 39 | 305 (1016)  |

*No significant differences were detected between exposed and control values collected during the same month (Student’s t-test, two-tailed).

**Table 6. Number of individuals with positive titers of MIC antibodies.**

| Antibody class | No. positive/total tested |
|----------------|---------------------------|
| IgA            | 0/87                      |
| IgE            | 3/128                     |
| IgG            | 8/118                     |
| IgM            | 7/24                      |

*IgE antibodies were determined by RAST and confirmed by RAST inhibition as described in the text. IgG, IgM, and IgA antibodies were detected using ELISA and confirmed by inhibition assay.

**Discussion**

Industrial experience with isocyanates has indicated that these chemicals can cause pulmonary hypersensitivity reactions and IgE antibody formation. Moreover, the antibody response appears to correlate with the degree of isocyanate exposure (1,2,5,16-18). These findings were observed in workers accidentally exposed to isocyanates and were reproduced in animal models of pulmonary and dermal isocyanate sensitivitvity (3,10-15). These results implied that severe exposure to an isocyanate such as MIC would also result in specific antibody production and perhaps pulmonary and/or dermal sensitization.

Animal studies employing MIC were required before clinical serologic evaluations could be initiated. The animal model enabled determination of whether MIC could function as a hapten, and whether the antibodies produced to MIC had haptenic specificity as opposed to...
Table 7. Antibody titer to MIC in serum samples collected following exposure.

| Individual | Date of sample | Total IgE (IU/mL) | Antibody titer by class |
|------------|----------------|-------------------|-------------------------|
|            |                |                   | IgM* | IgG | IgA | IgE% (% inhibition) |
| A          | 25 Jan 85      | 308               | ND   | Neg | Neg | 9.5 (37%)           |
|            | 15 Apr 85      | 242               | ND   | Neg | ND  | 2.7 (0%)            |
|            | 17 Jun 85      | 209               | ND   | Neg | Neg | 3.6 (0%)            |
| B          | 16 Jan 85      | 880               | 8    | >16 | Neg | ND                  |
|            | 17 Jan 85      | 803               | 4    | 32  | ND  | 6.9 (0%)            |
|            | 5 May 85       | 880               | Neg  | Neg | Neg | 4.6 (ND)            |

*ELISA was used to detect IgM, IgG, and IgA antibody. Specificity was confirmed by inhibition assay. Titers are the reciprocal of the highest serum dilution that gave a positive reading. ND = not determined; Neg = negative.

Table 8. Immunologic response in individuals with increasing severity of exposure.

| Severity of exposure | Antibody production (no. pos./no. tested) |
|----------------------|------------------------------------------|
|                      | IgM | IgG | IgA | IgE |
| Severe               | 22  | 1/7 | 2/20| 0/15| 4/22 |
| Moderate             | 41  | 4/10| 3/39| 0/31| 1/38 |
| Mild                 | 25  | 0/3 | 1/21| 0/17| 0/18 |

500,000 daltons; 64% of the primary amino groups were reacted with MIC). All binding with this conjugate was hapten-specific, as there was no binding of antibody to KLH. The hapten specificity of antibody implies that carriers other than serum albumin may have been involved in reaction with MIC.

Recent studies with 14C-TDI offer further indication of isocyanate binding with proteins other than serum albumin (25,26). In guinea pigs exposed to TDI by inhalation, the isocyanate was detected bound to a single protein of 66 K daltons, which was not serum albumin (25). Identification of this reactant protein should enable development of even more sensitive assays to detect isocyanate antibodies. Moreover, in view of the dependence of antibody production on exposure concentration (12,16–18), recognition of this protein reactant may provide an extremely sensitive method for biological monitoring to determine individual exposure to isocyanates.

The detection of antibody in this study was possible because of the exquisite sensitivity of RAST and ELISA assays. These tests can detect picogram to nanogram quantities of antibody. The antibody values reported here were low and tended to decline with time from exposure. For these reasons, clinical significance of such titers is doubtful. However, it should be noted that the detection of antibody with MIC specificity in Bhopal residents is the only irrefutable evidence that these individuals were exposed to methyl isocyanate and, consequently, isocyanate toxicity should be given prime consideration as the causative factor underlying the observed lesions.

This study was supported by grant ES01532 from the National Institute of Environmental Health Sciences. The authors thank E. Nigro for excellent preparation of the manuscript.

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