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Localization of inhaled trimellitic anhydride to lung with a respiratory lymph node antibody secreting cell response

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*Male Sprague-Dawley rats were exposed to trimellitic anhydride (TMA) by inhalation (500 $\mu\text{g}/\text{m}^3$), 4 hours a day, for 1 to 10 days. TMA was localized to lung cells by immunoelectron microscopy. Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis were used in an attempt to localize TMA to lung lavage proteins. The lung-associated lymph node (LALN) B-lymphocyte response was measured by quantitation of immunoglobulin (Ig)G, IgA, and IgM antibody secreting cells specific for TMA rat serum albumin (TM-RSA) by use of the enzyme-linked immunospot assay (ELISPOT) method. The IgG, IgM, and IgA antibody response to TM-RSA in serum and lavage fluid was quantitated by ELISA. Lung injury was assessed by the number of external lung hemorrhagic foci and lung weight. Immunoelectron microscopy localized TMA to alveolar and bronchial cells on all exposure days. ELISA detected trace amounts of TMA haptenized lavage proteins that could not be detected by Western blot analysis. A marked increase occurred in lung injury from day 7 to 10. The LALN IgG, IgA, and IgM antibody secreting cell response to TM-RSA paralleled measures of lung injury. IgG, IgM, and IgA serum and lavage antibody to TM-RSA were correlated with lung injury measures. Lavage and serum IgG antibody levels had the highest correlation with lung injury. (*J ALLERGY CLIN IMMUNOL* 1992;90:944-52.)*

Key words: Aerosol, anhydride, immune response, lung injury, occupation, hapten, ELISPOT

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Abbreviations used

TMA:	Trimellitic anhydride
ELISPOT:	Enzyme-linked immunospot assay
ELISA:	Enzyme-linked immunosorbent assay
LALN:	Lung-associated lymph node
TM-RSA:	Trimellityl conjugated rat serum albumin
ASC:	Antibody secreting cell
FAC:	Filtered air control
BAL:	Bronchoalveolar lavage
BCIP/NBT:	5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA:	Bovine serum albumin
PBS:	Phosphate-buffered saline
OD:	Optical density

A number of reactive, low molecular weight chemicals can cause respiratory disease in exposed industrial workers and populations.¹⁻³ The acid anhydrides, produced in billion pound quantities per year, cause a spectrum of occupational lung disease, which include pneumonitis and pulmonary hemorrhage.⁴ Lung disease caused by trimellitic anhydride (TMA) inhalation has been well characterized.⁵⁻⁷

An animal model of TMA lung disease has been developed in the Sprague-Dawley rat. Controlled TMA inhalation exposure results in defined histopathology and measures of lung injury that correlate with the immunologic response to TMA haptenized rat proteins.⁸⁻¹¹ The development of hemorrhagic lung lesions in this model was directly related to the TMA inhalation exposure concentration. The underlying lung disease was pneumonitis with pulmonary hemorrhage as the end result of interstitial inflammation. Neither lung lesions nor antibody responses were seen with trimellitic acid inhalation.⁸

The time-course of lung injury and immune response in this model has been explored.¹⁰ There was no lung injury or antibody response at 2 days and minimal lung injury at 6 days. At 10 days a marked increase occurred in hemorrhagic foci, lung weight, and in bronchoalveolar lavage (BAL) and serum antibody levels.¹⁰ Antibody levels in BAL were higher than the corresponding serum level and were highly correlated with measures of lung injury.¹²

These studies led to the consideration of a detailed hypothetical chain of events that could be verified by experiments to elucidate the complex pathogenesis of the extensive lung injury that is seen after 10 days of TMA inhalation (Table I).

The aims of this report were to determine the localization of TMA to respiratory tract cells by quantitative immunoelectron microscopy and to lung pro-

TABLE I. Hypothetical chain of events leading to TMA-induced lung injury

Phase	TMA inhalation
Sensitization (day 1-5)	TMA haptenized lung proteins/cells Diffuse/traffic with macrophages to LALNs
Immune response (day 5-10)	LALN immune response ASCs, antibody, cytotoxic T cells Antibody complexes with TMA haptenized lung proteins/cells
Immune injury Early (day 7)	Early cytotoxic (type II) lung injury by effector cells-macrophages/lymphocytes
Amplified (day 8-10)	Exponential increase in ASC, antibody, effector cell accumulation, and lung injury

teins by Western blot and enzyme-linked immunosorbent assay (ELISA) analysis of BAL fluid. In addition, the immunoglobulin (Ig)G, IgA, and IgM lung-associated lymph node (LALN), B-lymphocyte response was determined by the quantitation of antibody secreting cells (ASCs) specific for trimellityl conjugated rat serum albumin (TM-RSA) and correlated with the level of IgG, IgA, and IgM antibody to TM-RSA in serum and BAL fluid. These antibody responses were correlated with measures of lung injury.

MATERIAL AND METHODS

Exposure groups and inhalation system

Male Sprague-Dawley rats were exposed to micronized TMA powder (500 µg/m³) for 4 hours a day for 1 to 10 days. Each exposure day included five animals exposed to TMA and two exposed to filtered air control (FAC) that were killed immediately after the 4-hour exposure period.

Respirable-size particles of TMA (mass median aerodynamic diameter of approximately 2 µm, with >90% of the particles ≤10 µm) were aerosolized in a modified TSI fluidized Bed Aerosol Generator (Model 3400; TSI Inc., St. Paul, Minn.) and blown by means of a transvector Jet (Model 901B; Vortex Corp., Cincinnati, Ohio) into a 1000 L inhalation chamber. Details of the TSI modifications and inhalation system were previously described.⁹ The chamber concentration of TMA was measured during each exposure period by use of standard gravimetric methods.

Tissue pathology and lung lavage

Rats were anesthetized with sodium pentobarbital and exsanguinated. A necropsy was performed on all rats. The

thoracic block was removed, and all tissue, except a 1.5 cm portion of the trachea, was trimmed from the lungs. The lungs were weighed, and the number of external hemorrhagic foci on each lung lobe counted. With this dissection 4 to 5 paratracheal LALNs were obtained and transferred to cold phosphate-buffered saline (PBS) for subsequent ASC determination. The lungs were then lavaged or prepared for immunoelectron microscopy.

Lung lavage

A volume of 6 ml of PBS, pH 7.4, was slowly introduced into the lungs through the trachea. The saline-filled lungs were gently massaged, and the saline was collected from the lungs by inversion into a test tube. The BAL fluid was spun at 1000 rpm for 10 minutes to remove cells. The supernatant was frozen at -20°C for subsequent determination of specific BAL antibody levels and for detection of TMA haptenized BAL proteins.

The experimental animal studies met the requirements of the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication No. 86.23, revised 1985).

ELISA to detect TMA bound to BAL proteins

Lavage fluid from animals exposed to TMA (from 1 to 5 days) was analyzed by ELISA.¹² One tenth milliliter of BAL fluid in duplicate was added to 96-well microtiter plates for 18 hours at 4°C . Free absorption sites were blocked with 0.1 ml of 4% bovine serum albumin (BSA) for 1 hour at room temperature. A serum pool from rats exposed to TMA for 10 days, with extensive lung injury and a high titer of IgG antibody to TMA-RSA, was used as a probe to detect TMA haptenized proteins in lavage. Serum from rats exposed to FAC served as a control. Serial threefold dilutions (1:10 to 1:810) of this pool and control serum (0.1 ml diluted in 1% BSA in PBS) were added to each well and incubated in the cold for 18 hours. IgG antibody bound to TMA protein was detected with goat antirat IgG alkaline phosphatase, and color was developed with p-nitrophenyl phosphate.

ELISA antibody assays

TM-RSA was prepared as previously described.¹⁰ The conjugate has 10 to 20 TM groups per molecule of RSA.

IgG, IgA, and IgM antibodies to TM-RSA are determined by ELISA.¹⁰ In brief, polystyrene, 96-well plates (Dynatech, Alexandria, Va.) are coated with 0.1 ml TM-RSA (100 $\mu\text{g}/\text{ml}$ in bicarbonate buffer). After washing, free absorption sites are blocked with 4% BSA. One tenth milliliter of appropriate dilutions of serum or BAL fluid are added and allowed to react with TM-RSA coated wells for 18 hours at 4°C . For the detection of rat IgG antibody the plate is charged with alkaline phosphatase-conjugated goat antirat IgG (Sigma Chemical Co., St. Louis, Mo.) at a 1:500 dilution for 2 hours at room temperature. The color is developed with use of the substrate p-nitrophenyl phosphate, and the optical density (OD) is read at 405 nm with a

Dynatech, MicroELISA Autoreader. In a similar manner, IgM antibody was detected with alkaline phosphatase-conjugated goat antirat IgM (Cappel Laboratories, Cochranville, Pa.) at a dilution of 1:200. For the detection of rat IgA antibody, alkaline phosphatase-conjugated goat antirat IgA diluted 1:500 (Bioscience International; Kennebunkport, Maine) was used.

Antibody probes

A serum pool from rats exposed to TMA (10 days) was used as a probe for IgG to TM-proteins in these studies and had a titer to TM-RSA of 1.00 OD at a dilution of 1:900, with activity still present at a dilution 1:8100. A serum pool from humans exposed to TMA had an IgG titer to TM-HSA of 1.00 OD at a working dilution of 1:900 and activity was detectable at a 1:8100 dilution. This human pool was used in the immunoelectron microscopy studies.

Western blot analysis of BAL fluid proteins

Western blot was used for analysis of BAL fluid from rat lungs exposed to TMA (500 $\mu\text{g}/\text{m}^3$, 4 hours a day) for 1 to 10 days and from corresponding animals exposed to FAC. BAL fluid proteins collected on day 2, 7, 9, and 10 were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE 8% separating gel) under nonreducing conditions, transferred to nitrocellulose, and probed with the high titer rat serum containing IgG to TM-RSA at a 1:50 dilution. The blots were developed with goat antirat IgG alkaline phosphates (1:100), and color was developed with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Bio-Rad, Richmond, Calif.).

Immunoelectron microscopy

Excised lungs from animals exposed to TMA for 2, 4, and 10 days were filled with 6 ml fixative (1%) paraformaldehyde-1% glutaraldehyde via the trachea. After 2 hours each lung was cut into $1 \times 1 \times 0.5$ cm cubes, and 200 μm Vibratome (Technical Products International, Inc., St. Louis, Mo.) slices were prepared and rinsed overnight in cacodylate-HCl buffer. All studies were done as follows: triplicate slices were preincubated in 1% normal goat serum in PBS-1% BSA and then incubated with human anti-TMA (1:10) followed by goat antihuman IgG colloidal gold (1:25) in PBS-1% BSA. Normal human serum (1:10) was used as a control. Rat lung tissue exposed to TMA was run in parallel with lung tissue exposed to FAC as an additional control. After incubation with antihuman IgG colloidal gold, the tissues were postfixed for 2 hours in 1% osmium tetroxide, 1.5% potassium ferricyanide in 0.1 mol/L cacodylate-HCl buffer, dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812. Silver sections were cut on Reichert Ultracut E (Leica, Inc., Deerfield, Ill.) stained, examined, and photographed at $\times 10,000$ with a Hitachi H-600 TEM (Hitachi Medical Corp. of America, Tarrytown, N.Y.) (transmission electron microscope).

Samples were examined, photographed and analyzed in a blinded manner. A total of 30 micrographs from at least

2 blocks/sample (a maximum of 20 micrographs from one block) were printed at $\times 25,000$ and 20 micrographs from each sample were selected blindly for morphometric analysis with use of the R&M Biometrics (Nashville, Tenn.) Bioquant IV analysis system. The linear surface occupied by the plasma membrane in each micrograph was determined by digitizing morphometry with the Bioquant IV system and a Houston Hi-Pad digitizing tablet. The total number of colloidal gold particles per micrograph were counted directly. The ratio of colloidal gold particles to millimeter of plasma membrane was determined for each sample. The results were analyzed by the Bioquant IV system and results compared by means of the *t* test.

Enumeration of ASCs

The ELISPOT assay was used to quantitate ASCs from LALNs.¹³ Lymphocytes were obtained from the lymph nodes with disruption by teasing in 1% BSA-PBS, nylon mesh filtration, and concentration by centrifugation. The cell number was determined by counting in a Coulter (Coulter Electronics, Hialeah, Fla.) counter calibrated against hemocytometer counts. Milliliter HA plates (Millipore, Bedford, Mass.) were charged with TM-RSA (100 $\mu\text{g}/\text{ml}$) as antigen or RSA alone. Cells were added (serial twofold dilutions) from 3×10^5 to 1×10^4 in triplicate and placed in CO_2 incubator for 2 hours at 37°C . Spots were developed with alkaline phosphatase-conjugated goat antirat IgG (1:500), IgM (1:1000), and IgA (1:500) and color was developed with BCIP/NBT as substrate. The spots were read at $\times 40$ magnification with an American Optical (American Optical Scientific Instruments, Leica, Inc., Buffalo, N.Y.) eyepiece (AO 145) containing a square grid divided into 100 squares that facilitated counting of the spots.

Statistical procedures

Mean and standard error were calculated for all quantitative parameters. Statistical analyses on these data are performed with use of the Student's *t* test (RSI statistical package, BBN Research Systems, Cambridge, Mass.). In addition, Pearson's correlation statistic was determined on an IBM PCXT computer (IBM, Armonk, N.Y.) with the Epistat program.

RESULTS

ELISA detection of TMA bound to BAL proteins

TMA was localized to BAL proteins by ELISA with use of rat IgG antibody to TMA as a probe for TMA haptenized lavage proteins from exposed rats absorbed to the polystyrene wells. Fig. 1 demonstrates the elevated OD readings of lavage proteins from four rats exposed to TMA for 5 days. As the IgG anti-TMA antibody probe is diluted, the OD converges to match that of the control serum, representing background IgG protein in both lavage fluids. The differences in OD at dilutions 1:10, 1:30, and 1:90 were statisti-

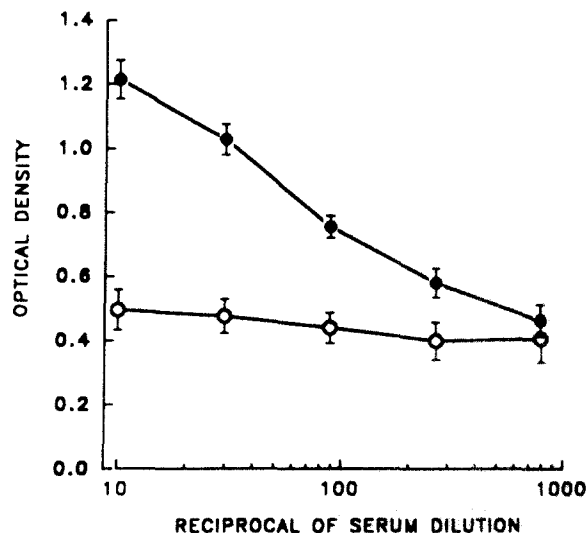


FIG. 1. Detection of TMA-haptenized proteins from lung lavage by ELISA. Difference in OD between the rat anti-TMA antibody curve ($\bullet$) and control serum curve ($\circ$) indicate the presence of TMA-haptenized proteins in the lavage fluid from animals exposed to TMA.

cally significant ($p < 0.001$). Day 5 was the only day that TMA haptenized proteins were detected.

Western blot analysis of lavage proteins

A representative SDS-PAGE analysis of lung lavage proteins from animals exposed to TMA for 2, 7, 9, and 10 days is shown in Fig. 2. With an increase in exposure days a significant increase occurred in the intensity of albumin and other proteins detected by Coomassie brilliant blue (Sigma Chemical Co., St. Louis, Mo.) staining (Fig. 2, *a*). In the Western blots (Fig. 2, *b*) only a single high molecular weight protein band representing IgG in the lavage was visible. Both the experimental (*top*) and control blots (*bottom*) developed by the goat antirat IgG secondary antibody showed increasing intensities of the IgG protein. However, no other TM-haptenized proteins were found in the experimental blot developed by the high-titer anti-TMA probe from animals exposed to TMA. The TM-RSA used as a positive control showed up clearly in the experimental (*top*) Western blot.

Immunoelectron microscopic detection of TMA

This technique gave a visual (Fig. 3) and a quantitative picture of TMA localized to alveolar (Table II) and bronchial cell surfaces with use of human IgG anti-TMA and goat antihuman IgG-colloidal gold to detect TMA. At all time points there were manyfold higher mean gold particles per millimeter of alveolar plasma membrane in lungs exposed to TMA devel-

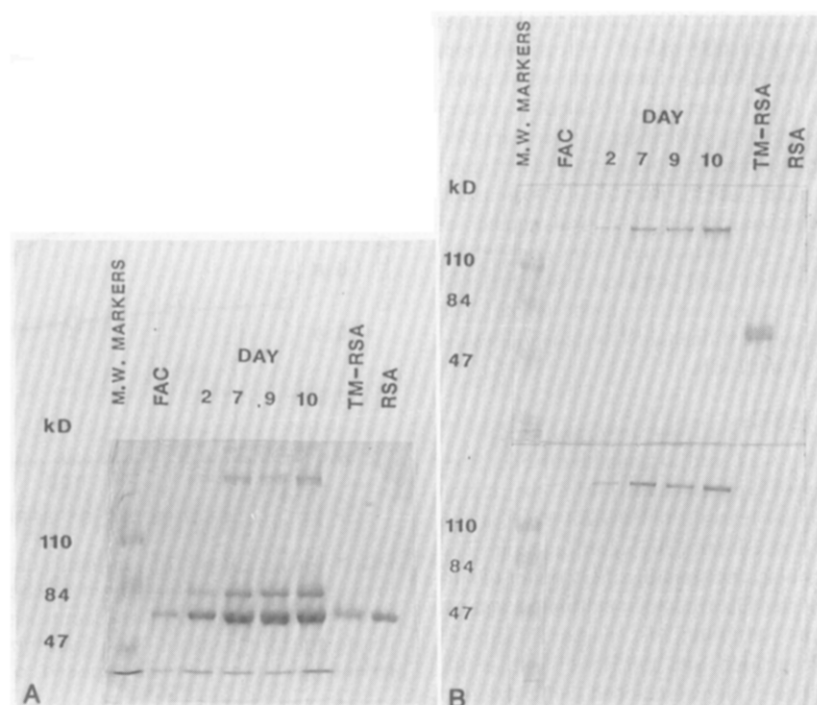


FIG. 2. A, SDS-PAGE of lung lavage proteins stained with Coomassie brilliant blue from animals exposed to TMA on day 2, 7, 9, and 10. FAC indicates lavage proteins from control animals exposed to filtered air. Increase in intensity and number of protein bands occurs as exposure days increase, especially days 7 to 10. TM-RSA is the TMA-haptenized rat serum albumin; RSA is rat serum albumin. **B,** Western blot analysis of lavage fluids in **A** developed with rat anti-TMA antibody (*top*) and (*bottom*) control serum. TM-RSA is clearly detected by the rat anti-TMA (*top*), whereas RSA alone, as expected, is not detected. High molecular weight band detected in both blots is IgG in the lavage fluid detected by the second antibody (goat anti-rat IgG). No other TMA-haptenized proteins are present.

TABLE II. Localization of TMA to alveolar tissue by immunoelectron microscopy

Day	Exposure	Antibody		
		Control*	Anti-TMA	
2	TMA	4.8 (0.20)†	59.1 (7.89)	$p < 0.01$
	FAC	3.2	7.0	
4	TMA	12.3 (1.07)	82.3 (10.5)	$p < 0.01$
	FAC	11.2	23.2	
10	TMA	54.1 (1.86)	382.7 (84.2)	$p < 0.01$
	FAC	18.8	22.3	

*Normal human serum as the control.

†Colloidal gold particles/linear millimeter of plasma membrane (SEM).

oped with anti-TMA as compared with the normal serum control (Table II). The normal human serum control showed a modest elevation of colloidal gold particles in lungs exposed to TMA at day 10 that represented endogenous rat IgG detected by the an-

tihuman IgG. Bronchial tissue exposed to TMA showed threefold increase in colloidal gold particles per millimeter plasma membrane on all exposure days in tissues treated with human anti-TMA as compared with tissues treated with the normal human serum

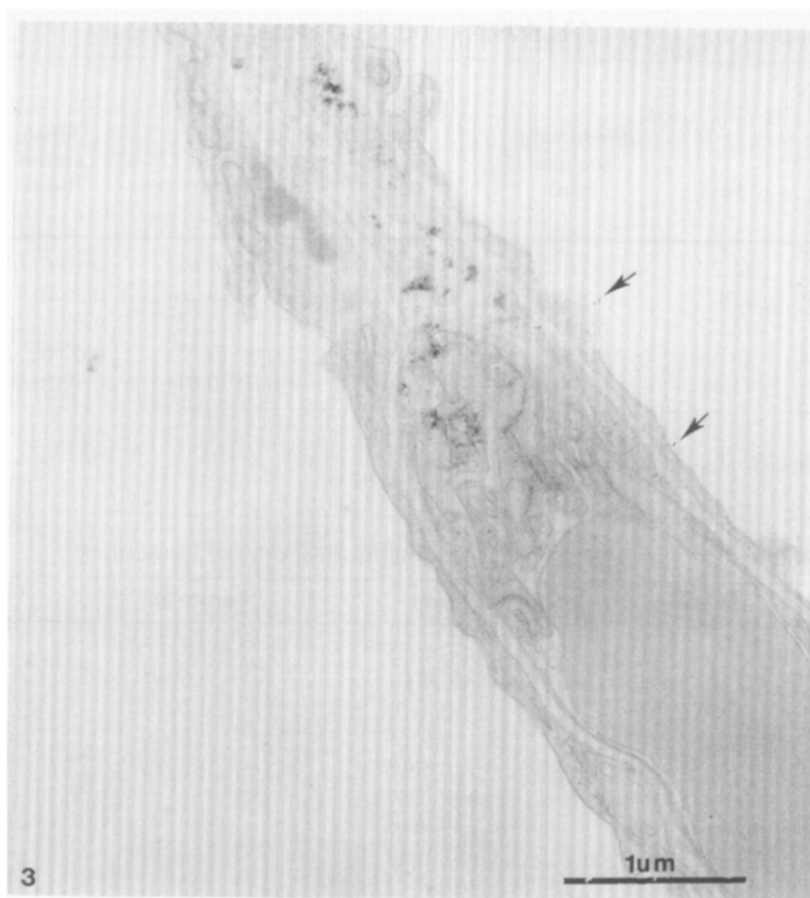


FIG. 3. Demonstration of TMA localized to alveolar cell surfaces by immunoelectron microscopy. Small black dots (*arrows*) represent colloidal gold particles. Cell-bound TMA was detected by human antibody to TMA followed by goat antihuman colloidal gold, 10 nm ($\times 25,000$).

TABLE III. Lung-associated lymph node ASC response

Day	IgG ASC	IgM ASC	IgA ASC
2	0	0	0
7	4219 (2908)	8648 (6782)	243 (243)
9	20955 (8390)	26643 (11893)	2306 (1185)
10	24758 (7338)	15416 (2728)	3407 (1527)

Mean (SEM) number of LALN ASCs by ELISPOT assay per 10^6 lymphocytes.

control. Lung tissue exposed to FAC had low levels of colloidal gold particles.

Antibody secreting cells (ASC) and antibody response

The enzyme-linked immunospot assay (ELISPOT) assay allowed the rapid quantitation of IgG, IgA, and IgM ASCs to TM-RSA from LALN. An accelerated increase occurred in lung injury from day 7 to 10 that was paralleled by an increase in IgG, IgA, and IgM

ASC number (Table III) and IgG, IgA, and IgM antibody to TM-RSA in serum and BAL fluid (Table IV). Animals exposed to FAC demonstrated no ASCs or detectable levels of IgG, IgA, or IgM antibody to TM-RSA in serum or BAL fluid. No antibodies or ASCs were detected, and no increase was seen in lung foci or weight with 2 days of TMA inhalation. Starting on day 7, there was the appearance of both an ASC and antibody response and an increase in measures of lung injury.

TABLE IV. Serum and lavage antibody response to TM-RSA related to lung injury

Day	Mean no.	Foci	Mean lung weight (gm)	IgG		IgM	
				Mean serum level	Mean lavage level	Mean serum level	Mean lavage level
				OD*		OD	
2	4	(1.2)	1.10 (0.02)	0.03 (0.01)	0.10 (0.02)	0.10 (0.02)	0.20 (0.04)
7	44	(28.7)	1.46 (0.09)	0.33 (0.11)	0.33 (0.16)	0.33 (0.11)	0.40 (0.11)
9	163	(62.7)	1.55 (0.08)	0.64 (0.14)	0.58 (0.12)	0.58 (0.12)	0.25 (0.10)
10	292	(31.8)	1.70 (0.09)	1.03 (0.18)	0.67 (0.15)	0.67 (0.15)	0.68 (0.27)
FAC	6	(1.1)	1.22 (0.04)	0.05 (0.03)	0.01 (0.01)	0.10 (0.01)	0.04 (0.01)

FAC, Filtered air control.

*Mean (SEM) level of antibody as measured by ELISA in OD units.

TABLE V. Relationships of immune response measures to measures of lung injury

	Foci	Weight	Serum Ig	Lavage Ig
ASC IgG	$r = 0.7822$ $p = 0.0001$	$r = 0.6925$ $p = 0.0001$	$r = 0.8471$ $p = 0.00001$	$r = 0.8457$ $p < 0.0001$
ASC IgM	$r = 0.4103$ $p = 0.0810$	$r = 0.4158$ $p = 0.0766$	$r = 0.5998$ $p = 0.0066$	$r = 0.2293$ NS
ASC IgA	$r = 0.6761$ $p < 0.0005$	$r = 0.5000$ $p = 0.0292$	$r = 0.5998$ NS	$r = 0.6674$ $p < 0.0001$
Serum IgG	$r = 0.9180$ $p = 0.0001$	$r = 0.8805$ $p < 0.0001$		$r = 0.9199$ $p < 0.0001$
Lavage IgG	$r = 0.9356$ $p < 0.0001$	$r = 0.8141$ $p < 0.0001$		
Serum IgM	$r = 0.8271$ $p < 0.0001$	$r = 0.8585$ $p < 0.0001$		$r = 0.7589$ $p = 0.0002$
Lavage IgM	$r = 0.6226$ $p < 0.0001$	$r = 0.6031$ $p < 0.0001$		
Serum IgA	$r = 0.6761$ $p = 0.0001$	$r = 0.5000$ $p = 0.0292$		$r = 0.28933$ NS
Lavage IgA	$r = 0.7643$ $p = 0.001$	$r = 0.7449$ $p = 0.0002$		
Weight	$r = 0.7838$ $p = 0.0001$			

*All r values are Pearsons' correlation coefficients.**Immune response and correlations with lung injury (Tables IV, V)**

Variables of immune response on day 2, 7, 9, and 10 were correlated with corresponding measures of lung injury from 19 animals (5 each on day 2, 9, and 10; and 4 on day 7) by use of Pearson's correlation.

IgG and IgA ASC number had a modest correlation with foci number and lung weight. A significant correlation occurred between IgG ASC number and serum and lavage IgG to TM-RSA. IgA ASC numbers were highly correlated with BAL IgA antibody to TM-RSA but not to serum level. IgM ASC were correlated

with serum IgM antibody to TM-RSA but not with BAL level.

Lavage IgG, IgA, and IgM antibody levels to TM-RSA were correlated with measures of lung injury as were serum antibody levels. IgG serum and lavage levels had the highest correlations with measures of lung injury. Lung weight and lung hemorrhagic foci were highly correlated.

Fig. 4 demonstrates these correlations with lung injury for IgG ASC number and IgG BAL antibody level to TM-RSA. The IgG antibody level in BAL was more closely related to hemorrhagic foci number than IgG ASC number.

IgA		
Mean serum level	OD	Mean lavage level
0.03 (0.01)		0.03 (0.01)
0.17 (0.03)		0.06 (0.05)
0.20 (0.04)		0.08 (0.02)
0.14 (0.03)		0.20 (0.05)
0.09 (0.05)		0.03 (0.01)

DISCUSSION

Little is known about how inhaled reactive chemicals interact with lung proteins and cells or the pathways that lead to an immune response and subsequent lung injury. In this report we have examined some of the steps (Table I) related to the induction of immunologic lung injury seen in this model. The first event examined was the binding of inhaled TMA to lung respiratory proteins and cells. Immunoelectron microscopy was very successful in localizing TMA to bronchial and alveolar plasma membrane surfaces. A marked increase occurred in the number of colloidal gold particles detected in lungs exposed to TMA as compared with control lungs during all exposure days examined. This technique measured TMA bound to cell surfaces and indicated that cell-bound TMA could serve as a target for subsequent immunocytotoxic injury. This was true even at 10 days, when despite high levels of endogenous antibody in the lung, TMA could still be detected by the human anti-TMA antibody probe (Table II).

The localization of TMA to BAL proteins was attempted with use of ELISA and Western blot technology. With a 4-hour inhalation exposure to TMA at 500 $\mu\text{g}/\text{m}^3$, only 24 μg of TMA would be inhaled, making detection a challenging problem. ELISA was able to detect TMA-haptenized lung lavage proteins from animals exposed to TMA only on day 5 of the examined days (days 1 to 5).

Western blot analysis led to several observations. As the number of exposure days increased, an increase occurred in the level of BAL protein on days 7, 9, and 10. This indicated that as the lung injury progressed, an increase occurred in leakage of proteins into the lung, which was supported by the increase in lung weight. Although ELISA was able to detect trace amounts of TM-protein in BAL on day 5, repeated trials with Western blot analysis did not show the presence of TM-haptenized proteins. These observations suggest that the Western blot method may not

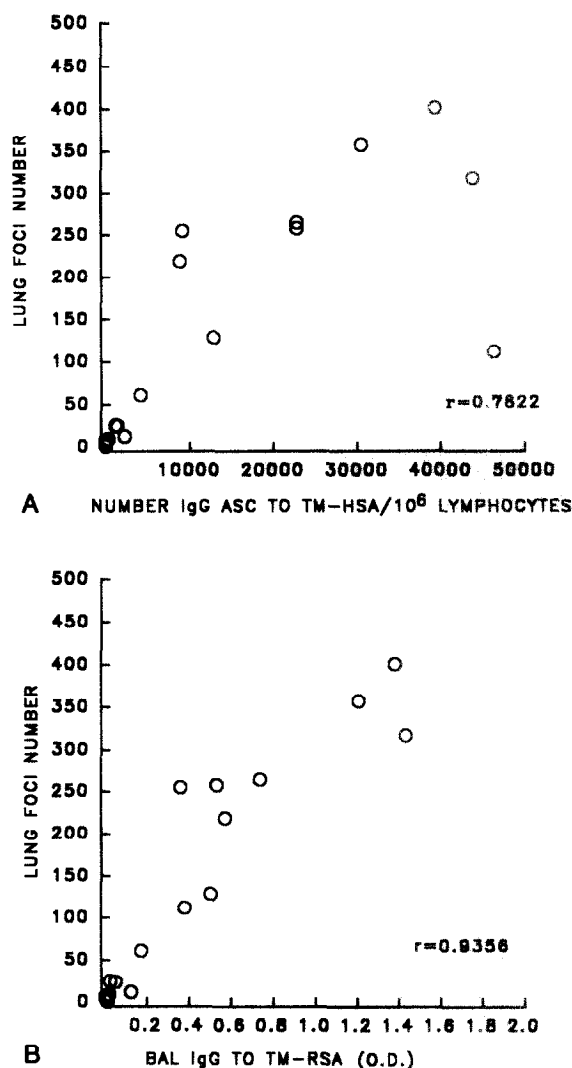


FIG. 4. A, Pattern and correlation of LALN IgG secreting cells to TM-RSA related to lung injury, hemorrhagic foci. A modest correlation occurs with lung injury ($r = 0.7822$, $p = 0.0001$). B, Close correlation ($r = 0.9356$, $p < 0.0001$) of IgG antibody to TM-RSA in BAL related to lung injury, hemorrhagic foci.

be sensitive enough to detect traces of haptenized proteins in BAL or more likely that TMA bound to respiratory blanket proteins is rapidly cleared from the lung by lung macrophages that carry TMA altered proteins to the respiratory lymph nodes. After an immune response has been mounted (day 6 to 10), the TMA-haptenized proteins may be complexed with antibody, phagocytosed by alveolar macrophages,⁸ and therefore are not detectable.

An acceleration of lung injury occurred from days 7 to 10 as measured by increased numbers of external lung hemorrhagic foci and lung weight. This accelerated lung injury was paralleled by an increase in IgG, IgA, and IgM ASC number from LALN. These cells are B lymphocytes and plasma cells that can be

detected in short-term culture by use of the ELISPOT method. These cells are identified by their production of antibody to the nitrocellulose-bound antigen, TM-RSA. There was no ASC LALN response in animals exposed daily to filtered air. A modest correlation was seen between IgG, IgA, and IgM LALN ASC number and lung injury (Table V and Fig. 4). This would be expected because this assay measures the intensity of the immune response to TMA-altered self proteins in the paratracheal LALNs, but is removed from the site of the lung injury. In this regard, IgG and IgA antibody to TM-RSA in BAL had the highest correlations with measures of lung injury. This was especially true for BAL IgG antibody (Table V).

These correlative data point to a role for IgG, IgA, and IgM antibody in the lung in the pathogenesis of injury. Further studies will be needed to determine the immunologic mechanisms of lung injury and the role of inflammatory cells in the generation of lung lesions.

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