Trimellitic Anhydride-Induced Allergic Response in the Guinea Pig Lung Involves Antibody-Dependent and -Independent Complement System Activation

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ABSTRACT
Trimellitic anhydride (TMA) is one of many low molecular weight compounds known to cause occupational asthma. In our previous studies the TMA-induced allergic response in guinea pigs was attenuated by depletion of complement. Specifically, the leakage of red blood cells and infiltration of inflammatory cells into the lung after TMA challenge was significantly reduced. Thus, we hypothesize that in the presence of specific antibody, TMA activates the complement system and complement activation products play a role in mediating inflammatory cell infiltration into the lung and lung hemorrhage. Guinea pigs were sensitized by intradermal injection of TMA in corn oil. An increase in the complement activation product C3a was detected in bronchoalveolar lavage, but not in plasma, of both sensitized and nonsensitized guinea pigs after intratracheal challenge with TMA conjugated to GPSA (TMA-GPSA). In vitro experiments demonstrated that TMA-GPSA caused complement activation by antibody-dependent as well as antibody-independent pathways. In sensitized animals, TMA-GPSA challenge caused significant increases in eosinophils, neutrophils, and macrophages in lung, along with increases in red blood cells and protein in the airspace. The infiltration of eosinophils was unique in that the magnitude of the GPSA/TMA-GPSA effect was significantly different between nonsensitized and sensitized animals. C3a concentrations in BAL correlated with all measures of cell infiltration in sensitized animals, but not in nonsensitized animals. These data indicate that complement activation in the absence of antibody is not sufficient for the complete allergic response to occur. Both sensitization and the complement system are required for TMA-induced eosinophilia.

Trimellitic anhydride (TMA) is one of many chemically reactive low molecular weight compounds with important industrial applications. It is widely used in the manufacture of paints, epoxy curing agents, printing inks and vinyl plasticizers. However, TMA is a respiratory sensitizer and is among the growing number of low molecular weight compounds known to cause occupational asthma. Humans exposed to TMA may experience immediate- and/or late-onset asthma (Zeiss et al., 1977; Hagmar et al., 1987). In guinea pig models, airway hyper-responsiveness, reversible airway obstruction, and airway inflammation, including significant eosinophilia, are characteristics of the TMA-induced allergic response (Hayes et al., 1992; Obata et al., 1992; Fraser et al., 1995).

Products of complement system activation, specifically the anaphylatoxins C3a and C5a, are known to cause chemotaxis of inflammatory cells, changes in vascular permeability, and bronchoconstriction (Regal, 1997a; Makrides, 1998). Because of these biological activities, products of complement activation are potentially important mediators of occupational asthma. Studies by others have shown that some allergens, such as grain dusts (Olenchock et al., 1978), house dust mite (Maruo et al., 1997), ragweed (Gonczi et al., 1997), and pli- catic acid (Chan-Yeung et al., 1980) activate the complement cascade in vitro. However, few in vivo studies have investigated the role of the complement system in occupational asthma. Leach et al. (1987) demonstrated a dose-related deposition of the third component of complement (C3) in the lung of rats sensitized and challenged with TMA dust. In our previous studies with TMA-sensitized guinea pigs, intratracheal instillation of TMA conjugated to guinea pig serum albumin (TMA-GPSA) induced an immediate and significant bronchoconstriction, decrease in circulating platelets, and increase in microvascular permeability. This was followed 24 h later with red blood cells in the airway and a significant infiltration of eosinophils, neutrophils, and mononuclear cells into the lung. However, if the guinea pigs were depleted of complement by cobra venom factor, the TMA-GPSA-in-

ABBREVIATIONS: TMA, trimellitic anhydride; GPSA, guinea pig serum albumin; BAL, bronchoalveolar lavage; RBC, red blood cells; EPO, eosinophil peroxidase; MPO, myeloperoxidase; YAC, yeast activated complement; SHEA, sheep erythrocytes coated with antibody.
duced leakage of red blood cells and infiltration of eosinophils and mononuclear cells was significantly reduced (Fraser et al., 1995). Thus, a role for the complement system in the TMA-induced allergic response is supported.

TMA and other low molecular weight chemicals are too small to stimulate the immune system but function as a hapten in complex with endogenous proteins to elicit antibody production. Both IgE and non-IgE mechanisms have been associated with occupational asthma induced by chemicals (Chan-Yeung and Malo, 1994). Passive transfer of human serum containing TMA-specific IgE to the airways of monkeys also transferred the asthmatic response, demonstrating a role for IgE in TMA-induced asthma (Dykewicz et al., 1988). However, IgE has not always been detected in individuals symptomatic for acid anhydride-induced respiratory disease (Zeiss et al., 1977; Rosenman et al., 1987; Nielsen et al., 1988), nor does IgE appear to be associated with late-onset occupational asthma (Grammer et al., 1998). Although TMA-specific IgE, IgG, IgM, and IgA have been measured in TMA-exposed workers and in animal models of TMA-induced respiratory disease, attempts to correlate effects of TMA with levels of TMA-specific antibody have been inconclusive (Zeiss et al., 1977; Sale et al., 1981). Low molecular weight chemicals such as toluene diisocyanate and formaldehyde can cause symptoms of asthma without sensitization (Chan-Yeung and Malo, 1994). Antibody-independent mechanisms leading to asthmatic responses need further consideration.

We hypothesize that in the presence of TMA-specific antibody, TMA activates the complement system and complement activation products play a role in mediating the response of inflammatory cell infiltration into the lung and lung hemorrhage. Our previous studies of TMA-induced allergic responses in complement-depleted guinea pigs support this hypothesis (Fraser et al., 1995). Because of the potential for various low molecular weight chemicals to cause antibody-independent responses in the lung and to activate the complement system, the present studies were designed to examine events in sensitized as well as nonsensitized animals. Thus, to further test the hypothesis, the purpose of the present study was to 1) determine whether complement system activation can be detected after TMA-GPSA challenge; and 2) determine whether sensitization is required for TMA-GPSA-induced complement activation, cell infiltration, and lung hemorrhage.

Materials and Methods

In Vivo Experiments

TMA Sensitization and Challenge. Details of the guinea pig model of the TMA-induced allergic response appear in Fraser et al. (1995). All animal studies were approved by the University of Minnesota Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory animals as adopted and promulgated by the U.S. National Institutes of Health. Female Hartley guinea pigs (n = 63; Charles River Laboratories, Portage, MI) weighing 279 ± 3 g were sensitized with either a single 100-µl intradermal injection of 0.3% TMA (w/v in corn oil) on day 1 or three 100-µl intradermal injections of 30% TMA given on days 1, 3, and 5. Additional animals were given a single 100-µl intradermal injection of corn oil as a control for the vehicle and are referred to as nonsensitized animals. Three weeks after sensitization (average weight 426 ± 5 g) the guinea pigs were anesthetized with ketamine and xylazine, bled by cardiac puncture to obtain EDTA plasma, and then given intratracheally either 40 µl (4 mg) of GPSA as a control or 40 µl (4 mg) of TMA conjugated to guinea pig serum albumin (TMA-GPSA) as a challenge. To reduce the potentially fatal immediate histamine-induced bronchoconstriction upon TMA challenge, guinea pigs were given 6.1 mg/kg i.p. of the H1 antagonist pyrilamine 30 min before challenge. TMA was conjugated to GPSA as described previously (Fraser et al., 1995). The degree of substitution was 18 to 21 moles of TMA per mole of GPSA.

Experimental Groups. In experimental group 1, 24 h after intratracheal instillation with either GPSA or TMA-GPSA, the guinea pigs were anesthetized with pentobarbital, serum and EDTA plasma were collected by cardiac puncture, the lungs were lavaged with six 5-ml aliquots of phosphate-buffered saline, and the lungs were removed for analysis. Six treatment groups were considered in this study: nonsensitized animals, and 0.3% TMA- and 30% TMA-sensitized animals, each intratracheally instilled with either GPSA or TMA-GPSA. Each treatment group contained 10 to 13 animals except for the nonsensitized GPSA control group in which six animals were used. The following responses were measured: cellular infiltration into the lung and bronchoalveolar lavage (BAL), total protein and red blood cells (RBC) in the BAL, and the complement activation product C3a in the BAL and plasma. For an additional control, four nonsensitized animals and four animals sensitized with 0.3% TMA were not subjected to intratracheal instillation. However, the animals were lavaged as previously described and the same responses measured.

Experimental group 2 was treated identically as the first group except only 0.3% TMA-sensitized animals were used. The same responses were examined at 6 and 48 h after challenge with five to seven animals per group. Four treatment groups were considered in this study: 0.3% TMA-sensitized animals, each intratracheally instilled with either GPSA or TMA-GPSA, with lavage either 6 or 48 h after challenge.

In experimental group 3, animals were either sensitized with 0.3% TMA or nonsensitized. Twenty-four hours after intratracheal instillation with either GPSA or TMA-GPSA, the guinea pigs were anesthetized with pentobarbital and lung edema was measured as outlined below. Animals were not lavaged. Four treatment groups were considered in this study: nonsensitized or 0.3% TMA-sensitized animals each intratracheally instilled with either GPSA or TMA-GPSA.

Cell Infiltration. Total white blood cells in BAL fluid were counted by standard methods in a hemacytometer. Cytospin preparations of BAL cells (3 × 104 cells) were made using a Shandon Cytospin 3 centrifuge (Shandon Lipshaw Inc., Pittsburgh, PA). Cells were stained with a modified Wright's stain (Diff Quik; American Scientific Products, McGraw Park, IL) and at least 200 cells were counted and categorized as neutrophils, eosinophils, macrophages, or lymphocytes as determined by their morphology. Eosinophils and neutrophils in the BAL fluid were also measured by assaying the eosinophil peroxidase (EPO) activity and the myeloperoxidase (MPO) activity, respectively, of the cells within the BAL fluid. Lung lobes were processed as previously described (Fraser et al., 1995) for the measurement of EPO and MPO activity as an estimate of the number of eosinophils and neutrophils, respectively.

Total Protein in the BAL. Total protein in the BAL fluid was measured using the method of Lowry et al. (1951).

RBC in the BAL. RBC in the BAL, an indicator of lung hemorrhage, were assessed by measuring the absorbance at 412 nm (hemoglobin) of an aliquot of the BAL cell pellet in which the cells were lysed by freeze-thawing (Fraser et al., 1995).

Determination of C3a in BAL Fluid and Plasma. BAL fluid used for C3a analysis was first concentrated approximately 10× using Centricon 3 concentrators (Amicon, Inc., Beverly, MA). The assay for guinea pig C3a was based on the Western blot technique of Maeno et al. (1992) and was modified from our previous studies (Begal and Klos, 1999). C3a in either 2 µl of plasma or 40 µl of...
concentrated BAL fluid was separated from intact C3 by SDS-polyacrylamide gel electrophoresis under denaturing conditions (Laemmli, 1970) using a 20% acrylamide gel. Proteins on the gel were electrophotographically transferred to a 0.2-μm nitrocellulose membrane (BA-S 83; Schleicher & Schuell, Keene, NH). The primary antibody used for immunodetection was the IgG fraction of a rabbit polyclonal antibody to the nine carboxyl-terminal amino acids of guinea pig C3α. The nitrocellulose blot was successively incubated in 3% bovine serum albumin (overnight at 4°C), 1:1000 dilution of the primary antibody (anti-C3α-peptide antibody; 2 h at 25°C), 1:15,000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase (Pierce, Rockford, IL; 1 h at 25°C), and chemiluminescence detection reagents (ECL Plus; Amersham Pharmacia Biotech Inc., Piscataway, NJ; 5 min at 25°C). Images of light emission were recorded on X-ray film, digitized, and quantified by densitometric analysis using a PC and Scion Image for Windows (public domain NIH Image program developed at the U.S. National Institutes of Health). A standard pool of yeast-activated complement (YAC) was prepared by activating pooled guinea pig serum with yeast cell walls. YAC was used to construct a standard curve and regression equation for each Western blot. C3α was expressed as YAC equivalents: the microliters of YAC giving a signal equivalent to the 2 μl of unknown plasma or 40 μl of concentrated BAL fluid loaded onto the gel. YAC equivalents in the BAL fluid were additionally adjusted for the degree of sample concentration.

**Lung Edema.** Lung edema after challenge was determined by the wet/dry weight of lungs not subjected to lavage. Twenty-four hours after intratracheal instillation of GPSA as a control or TMA-GPSA as a challenge, the lungs were removed and the wet weights determined. Dry weights were determined after heating the lungs to constant weight at 80°C for 72 h.

**Statistical Methods.** All data were log transformed to equalize variances. Figures show the geometric mean ± 1 standard error, with significant comparisons indicated by an asterisk. Statistical significance was defined as p < 0.05. Statistical analyses were done using JMP and SAS software (SAS Institute Inc., Cary NC).

In experimental group 1 with 6 treatment groups, three different analyses were conducted on data from the BAL and lung. First, GPSA control and TMA-GPSA challenge within each sensitization group were compared by ANOVA with one-tailed single degree of freedom contrasts (short brackets in Figs. 1 and 4–7). Second, to determine whether the GPSA control values for the variables changed between the nonsensitized, 0.3% TMA- and 30% TMA-sensitized groups, a one-way ANOVA was used (long brackets in Figs. 1 and 4–6). Third, two-way ANOVA with one-tailed single degree of freedom contrasts was used to test for effects of different sensitization levels on the magnitude of the GPSA/TMA-GPSA effect (Table 1).

In experimental group 1, the pre- and postinstillation C3α concentrations in plasma were compared by paired t tests (short brackets in Fig. 2). These changes in pre- to postinstillation values were then compared between GPSA control and TMA-GPSA challenge within sensitization groups by t tests (longer brackets in Fig. 2).

**In Vitro Experiments**

**Complement Hemolytic Activity.** The ability of TMA-GPSA to inhibit the hemolytic activity of the classical complement pathway was assessed in vitro. Normal guinea pig serum (Advanced Research Technologies, Inc., San Diego, CA) served as a source of complement. Sheep erythrocytes coated with antibody (SHEA) were obtained from Sigma Chemical Co. (St. Louis, MO). Enzyme-linked immunosorbent assay analysis verified that the normal guinea pig serum did not contain TMA-GPSA specific IgG1 or IgG2 (Fraser et al., 1998). Normal guinea pig serum was initially incubated (90 min at 37°C) with varying concentrations of TMA-GPSA or GPSA in a reaction volume of 150 μl. Then 50 μl SHEA (2 × 10⁸ cells/ml) were added and incubation continued at 37°C for 60 min. The incubation mixture was centrifuged and absorbance of the supernatant at 415 nm was measured and used as an indicator of the ability of any remaining complement to lyse the SHEA. All dilutions were made with veronal buffer containing 0.1% gelatin, 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 5% dextrose (Gewurz et al., 1967).

**Measurement of C3 Conversion.** The C3 molecule is cleaved upon complement activation into the fragments C3a and C3b, with subsequent degradation of C3b to iC3b, C3dg and C3d. These fragments resulting from activation and degradation of C3, i.e., C3 conversion products, were measured in vitro as an indicator of complement system activation induced by TMA-GPSA in the presence or absence of antibody. Normal guinea pig serum (Advanced Research Technologies, Inc., San Diego, CA) served as a source of complement without antibody. The absence of TMA specific IgG1 or IgG2 antibody in the normal guinea pig serum was confirmed by enzyme-linked immunosorbent assay (Fraser et al., 1998). Serum obtained from guinea pigs immunized with 0.3% TMA in corn oil served as a source of complement containing TMA-specific antibody. TMA-GPSA or GPSA of varying concentrations was incubated (15 min at 37°C) with an equal volume of complement in the presence or absence of antibody. C3 conversion was assessed using immunofixation techniques as described by Strong and Watkins (1979) and as we have previously described (Regal et al., 1993). Briefly, after incubation with TMA-GPSA or GPSA, 1-μl samples of complement were electrophoresed on agarose gels using barbital buffer containing EDTA. After electrophoresis, C3 and C3 fragments were detected by immunoprecipitation with the IgG fraction of goat anti-guinea pig C3 (Cappel/ICN Pharmaceuticals, Inc; Aurora, OH). This antibody will recognize C3 and the large fragments generated during C3 cleavage. The precipitate was stained with Coomassie blue, and the density of the C3 conversion products was digitized and quantified by densitometric analysis using a PC and Scion Image for Windows (public domain NIH Image program developed at the U.S. National Institutes of Health). A sample of YAC served as a positive control for C3 conversion.

**Statistical Methods for C3 Conversion.** Four experimental groups were considered: incubation with or without antibody with varying concentrations of either GPSA or TMA-GPSA. The following comparisons were made by two-way ANOVA considering concentration and the group: GPSA with antibody versus GPSA without antibody, TMA-GPSA with antibody versus TMA-GPSA without antibody, GPSA with antibody versus TMA-GPSA without antibody, GPSA without antibody versus TMA-GPSA without antibody (Fig. 3).

**Results**

**Does TMA-GPSA Cause Complement Activation?**

**In Vivo Studies.** Our previous studies using complement-depleted guinea pigs indicated the complement system plays an important role in mediating TMA-GPSA-induced increases in the number of eosinophils, mononuclear cells, and RBC in BAL (Fraser et al., 1995). If complement activation products mediate the response, complement system activation should be detectable in vivo in the lung coincident with the TMA-GPSA-induced response. Therefore, as an indicator of local complement activation, we measured the concentration of C3α in BAL 24 h after intratracheal instillation of either GPSA as a control or TMA-GPSA as a challenge. Both nonsensitized guinea pigs and animals sensitized with either 0.3% TMA or 30% TMA were used in these experiments. As shown in Fig. 1, in nonsensitized animals and in animals sensitized with 0.3% TMA, the C3α concentration in the BAL 24 h after challenge with TMA-GPSA was significantly greater than in control animals given GPSA. In guinea pigs sensitized with 30% TMA an increase in C3α concentration after TMA-GPSA challenge relative to GPSA control animals was seen but was not significant (p = 0.06). C3α concentra-
tions in the BAL of GPSA-challenged animals were not significantly different comparing nonsensitized, 0.3% TMA- and 30% TMA-sensitized animals.

Systemic complement activation was assessed by measuring C3a concentrations in plasma (Fig. 2). C3a was measured in plasma collected immediately before (pre) and 24 h after (post) instillation of GPSA control or TMA-GPSA challenge. Paired t tests were conducted to compare the pre- and postinstillation data within each treatment group. Plasma C3a concentrations decreased significantly from pre- to postinstillation in guinea pigs sensitized with 0.3% TMA, but not in the other treatment groups. Within each sensitization group, the change between pre- and postinstillation C3a concentrations comparing GPSA versus TMA-GPSA-challenged animals was not significant by t test. These data indicate that the pre- to postinstillation changes are no different whether animals are intratracheally instilled with either GPSA or TMA-GPSA. Changes in plasma C3a concentrations (pre- to postinstillation) were not directly related to concentrations of C3a in the BAL as indicated by least-squares regression (data not shown). In addition, plasma volumes as indicated by hematocrit measurements did not change significantly from pre- to postinstillation (data not shown).

To determine the time after challenge at which maximal complement activation occurred, C3a concentrations in the BAL and plasma were also measured in experimental group 2 at 6 and 48 h after instillation of either GPSA or TMA-GPSA. Compared with GPSA control animals, C3a concentrations in the BAL after TMA-GPSA challenge were significantly elevated at 6 but not at 48 h (data not shown). In plasma, the C3a concentrations were not different between GPSA control and TMA-GPSA-challenged animals at either 6 or 48 h after instillation (data not shown).

In Vitro Studies. Since studies in vivo indicated that activation of the complement system in BAL occurred with TMA-GPSA challenge, we examined the ability of TMA-GPSA to affect the complement system in vitro. Complement activation via the classical pathway is measured by the ability of complement to lyse SHEA. Preliminary experiments indicated that a 1:600 dilution of guinea pig serum caused lysis of approximately 50% of the SHEA. Thus, a 1:600 dilution of normal guinea pig serum was incubated with varying concentrations of TMA-GPSA or GPSA (0.3 to 300 μg/ml) as described under Materials and Methods. SHEA were then added to assess the complement hemolytic activity remaining. The ability of normal guinea pig serum to lyse SHEA was not affected by incubation with GPSA at concentrations from 0.3 to 300 μg/ml. However, after normal guinea pig serum was incubated with as little as 0.3 μg/ml TMA-GPSA, SHEA were not lysed. Lower concentrations of TMA-GPSA did not affect the hemolytic ability of normal guinea pig serum. These data indicate that TMA-GPSA affects the ability of the complement pathway to lyse SHEA.

To investigate this phenomenon further and more closely examine the importance of antibody in the activation of complement in vivo, we examined the ability of GPSA and TMA-GPSA to cause C3 conversion in vitro both in the presence and absence of TMA-specific antibody. As shown in Fig. 3, incubation of guinea pig serum with TMA-GPSA resulted in significantly more C3 conversion than incubation with GPSA, both in the presence and absence of TMA-specific antibody. In addition, after incubation with TMA-GPSA the density of C3 conversion products was significantly greater in the presence of antibody than without antibody. Conversion of C3 was minimal after incubation with GPSA, whether in the presence or absence of antibody. Thus, TMA-GPSA, but not GPSA, was causing cleavage of the third component of complement in vitro, consistent with the ability of TMA-GPSA challenge to cause complement activation in the BAL in vivo.

**Does TMA-GPSA Induce Cell Infiltration and Increased Protein and Numbers of RBC in Both Nonsensitized and Sensitized Guinea Pigs?** Our previous studies using sensitized guinea pigs documented infiltration of inflammatory cells into the lung and increased protein and numbers of RBC in the BAL after TMA-GPSA challenge (Fraser et al., 1995). However, the effect of TMA-GPSA in nonsensitized guinea pigs had not been rigorously compared with that in sensitized animals. We examined this question in experimental group 1 using two different types of statistical analysis. First, we tested for significant GPSA versus TMA-GPSA effects using ANOVA with single degree of freedom contrasts. These comparisons are shown by the short brackets in Figs. 4 through 6. As shown in Fig. 4, in sensi-
tized guinea pigs, the numbers of eosinophils, neutrophils, and macrophages in the BAL significantly increase after TMA-GPSA challenge compared with the GPSA control. However, this effect was not significant in nonsensitized animals. Eosinophils and neutrophils in the BAL were also assessed by determining EPO and MPO activity, respectively, and statistical findings were identical to those using the counts of eosinophils and neutrophils (data not shown).

Numbers of lymphocytes in the BAL were not significantly different between TMA-GPSA-challenged and GPSA control animals (data not shown). Eosinophils and neutrophils in lung tissue, shown in Fig. 5, were assessed by measuring EPO and MPO activity, respectively. EPO activity in the lung tissue is significantly increased after TMA-GPSA challenge compared with the GPSA control in animals sensitized with 30% TMA, but not in 0.3% TMA-sensitized nor in nonsensitized animals. MPO activity in lung tissue is significantly elevated after TMA-GPSA challenge compared with the GPSA control in both groups of sensitized guinea pigs, but not in nonsensitized animals.

Total protein and numbers of RBC in the BAL, shown in Fig. 6, both increased significantly 24 h after TMA-GPSA challenge compared with the GPSA control in sensitized guinea pigs, but not in nonsensitized animals.

The second type of statistical analysis of experimental group 1 was designed to determine whether the GPSA versus TMA-GPSA effect differed between nonsensitized, 0.3% TMA- and 30% TMA-sensitized animals. ANOVA with single degree of freedom contrasts was used to test for differences in GPSA versus TMA-GPSA effects. A significant interaction ($p < 0.05$) indicates that the magnitude of the GPSA versus TMA-GPSA effect varies between the different sensitization groups. As shown in Table 1, the difference in eosinophils in the BAL between GPSA control and TMA-GPSA-challenged animals varied significantly comparing nonsensitized guinea pigs with either 0.3% TMA- or 30% TMA-sensitized guinea pigs. The difference in lung EPO between GPSA control and TMA-GPSA-challenged animals comparing nonsensitized to 30% TMA-sensitized animals was also significantly different.

These significant differences in the magnitude of the change between GPSA control and TMA-GPSA challenge between the different sensitization groups suggest that the numbers of eosinophils observed in the BAL and lung in response to TMA-GPSA challenge are dependent upon sensitization. In contrast, for all other variables, the GPSA versus TMA-GPSA effect did not differ comparing nonsensitized and sensi-

![Fig. 3](image1.jpg)

**Fig. 3.** In vitro conversion of C3 by GPSA or TMA-GPSA. GPSA or TMA-GPSA was incubated with complement in the presence (+Ab) or absence (−Ab) of TMA-specific antibody. The density of the C3 conversion products was determined as described under Materials and Methods. Values represent the mean ± S.E. of density values from three to six separate experiments. *$p < 0.05$; treatment groups were compared by two-way ANOVA. ns, not significant.

![Fig. 4](image2.jpg)

**Fig. 4.** Total numbers of eosinophils, neutrophils, and macrophages in BAL of nonsensitized, 0.3% TMA- and 30% TMA-sensitized animals 24 h after intratracheal instillation of GPSA or TMA-GPSA. *$p < 0.05$; ns, not significant.

## Table 1

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<th>Sensitization</th>
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<th>30% TMA</th>
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* Data were log transformed before analysis. ANOVA with one-tailed single degree of freedom contrasts.

* $p < 0.05$. 
sitized animals. This suggests that these responses are not dependent on sensitization. Differences in the GPSA versus TMA-GPSA effects were not detected comparing 0.3% TMA- to 30% TMA-sensitized animals (data not shown).

To determine whether the instillation procedure itself markedly altered the cell populations in the lung, a limited number of nonsensitized \((n = 4)\) and 0.3% TMA-sensitized animals \((n = 4)\) were lavaged, but not intratracheally instilled. The data were log transformed to equalize variances and ANOVA with two-tailed single degree of freedom contrasts was used to compare the response of GPSA-instilled animals in experimental group 1 with these noninstilled animals. Minor but statistically significant differences in the number of macrophages and red blood cells in the BAL were observed for both nonsensitized and sensitized groups. For example, in 0.3% TMA-sensitized guinea pigs, the total number of macrophages recovered in the BAL of noninstilled guinea pigs was 339 to 521 \(\times 10^4\) cells (95% confidence interval) compared with the 613 to 773 \(\times 10^4\) cells recovered in the BAL of GPSA-instilled animals. Similar differences in the number of macrophages were seen in nonsensitized guinea pigs. OD\(_{412}\) of the BAL, which reflects the number of RBC in the BAL, increased from 0.07 to 0.10 absorbance units (95% confidence interval) in 0.3% TMA-sensitized, noninstilled animals to 0.15 to 0.23 (95% confidence interval) in 0.3% TMA-sensitized, GPSA-instilled animals. Again, similar results were observed in nonsensitized animals. In addition to the changes observed for macrophages and red blood cells in the BAL, statistically significant differences in the EPO activity in the lung of 0.3% TMA-sensitized guinea pigs was seen between noninstilled and GPSA-instilled animals.

EPO activity in the lung of noninstilled animals (244–448 OD/min/g of dry lung, 95% confidence interval) was slightly greater than in GPSA-instilled animals (83–220 OD/min/g of dry lung, 95% confidence interval). In nonsensitized animals, EPO activity in the lung did not differ between noninstilled and GPSA-instilled animals. Statistically significant differences between noninstilled and GPSA-instilled animals were not detected for the other variables.

The TMA-GPSA-induced response in the lung in experimental group 2 was examined at 6 and 48 h after instillation of GPSA or TMA-GPSA (data not shown). Compared with GPSA control animals, the numbers of eosinophils, neutrophils, and macrophages in the BAL were not significantly increased after TMA-GPSA challenge at either 6 or 48 h after challenge. Total protein and numbers of RBC in the BAL were significantly increased at 6 h, but not at 48 h after TMA-GPSA challenge compared with the GPSA control. Neither EPO nor MPO activity in the lung tissue were significantly different between TMA-GPSA-challenged and GPSA control animals at 6 h, but MPO was elevated at 48 h after challenge with TMA-GPSA compared with the GPSA control. Thus, the peak of the response is at 24 h with some changes in protein and RBC in the BAL occurring earlier at 6 h after challenge, and changes in MPO activity in the lung tissue remaining at 48 h after challenge.

**Effect of Sensitization on Resident Inflammatory Cells.** Examination of the data in experimental group 1 suggested that sensitization itself had an effect on the number of cells in the BAL and lung tissue and on the concentra-
tion of C3a in the BAL. Differences due to sensitization alone were tested by one-way ANOVA of the response in GPSA control animals. Numbers of eosinophils in the BAL (Fig. 4) and EPO and MPO activity in lung tissue (Fig. 5) differed between nonsensitized, 0.3% TMA- and 30% TMA-sensitized groups of animals. Differences in GPSA control animals were not detected for the other variables.

**Does the Extent of Complement Activation Predict the Magnitude of the Allergic Response?** If products of complement activation mediate the TMA-induced allergic response, we would expect the extent of complement activation to predict the magnitude of the response. Correlation of C3a concentrations in the BAL with the response determined 24 h after intratracheal instillation of GPSA or TMA-GPSA is shown in Table 2. In nonsensitized animals, C3a did not significantly correlate with any of the variables except the total protein in the BAL. For sensitized animals, a significant correlation with C3a was detected for all variables except lung MPO activity. These data are consistent with a role for complement in mediating the TMA-GPSA-induced response in sensitized guinea pigs.

**Does TMA-GPSA Cause Lung Edema in Sensitized and Nonsensitized Animals?** In experimental group 3, lung edema was assessed 24 h after intratracheal instillation of GPSA or TMA-GPSA by determining the wet/dry weights of the lung tissue. The lungs of these animals were not lavaged. The GPSA control groups in nonsensitized versus 0.3% TMA-sensitized animals were not significantly different as determined by one-way ANOVA. As shown in Fig. 7, the wet/dry weights increased significantly after TMA-GPSA challenge in sensitized guinea pigs, but not in nonsensitized animals. However, as shown in Table 1 (p = 0.33), the magnitude of the GPSA versus TMA-GPSA effect was not significantly different between the sensitization groups.

**Discussion**

The TMA-induced allergic response in the guinea pig includes immediate bronchoconstriction, influx of inflammatory cells into the lung with significant eosinophilia, increased microvascular permeability, and hemorrhage (Fraser et al., 1995). Complement anaphytoxins C3a and C5a are potential mediators of the TMA-induced response in the lung because they are both known to cause bronchoconstriction; be chemotactic for inflammatory cells, including eosinophils.

**TABLE 2**

<table>
<thead>
<tr>
<th>Cell infiltration into airspace</th>
<th>Nonsensitized</th>
<th>Sensitized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td>0.16</td>
<td>0.62*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.50</td>
<td>0.70*</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.32</td>
<td>0.58*</td>
</tr>
<tr>
<td>Cell infiltration into the lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung EPO</td>
<td>-0.02</td>
<td>0.47*</td>
</tr>
<tr>
<td>Lung MPO</td>
<td>0.18</td>
<td>0.14</td>
</tr>
<tr>
<td>RBC in BAL</td>
<td>0.46</td>
<td>0.61*</td>
</tr>
<tr>
<td>Protein in BAL</td>
<td>0.62*</td>
<td>0.75*</td>
</tr>
</tbody>
</table>

a Values are Pearson correlation coefficients. Data were log transformed before analysis.

b Includes 0.3% TMA- and 30% TMA-sensitized animals.

c p < 0.05.

Studies by Leach et al. (1987) in the rat demonstrated increased deposition of C3 in the lung coincident with TMA-induced hemorrhagic lung foci. Our previous studies of the TMA-induced allergic response in guinea pigs depleted of complement provided evidence for an important role of the complement system in the numbers of eosinophils, mononuclear cells, and red blood cells in the airspace following TMA-GPSA challenge (Fraser et al., 1995). Our current studies have demonstrated increased concentrations of C3a in the BAL before, or coincident with, increased cell infiltration in the lung after TMA-GPSA challenge. These data provide additional evidence supporting the hypothesis that TMA activates the complement system and complement activation products play a role in mediating the response of inflammatory cell infiltration into the airspace and lung hemorrhage.

Sensitization clearly was not required for TMA-GPSA-induced complement activation in vivo. Increased C3a concentrations in the BAL were observed after TMA-GPSA challenge in nonsensitized as well as sensitized animals. An antibody-independent mechanism by which TMA-GPSA activates the complement system was therefore suggested. In vitro experiments measuring C3 conversion confirmed antibody-independent activation of the complement system. This is the first demonstration that TMA conjugated to protein can activate the complement system in the absence of antibody. However, the in vitro experiments also indicated that in the presence of antibody, complement activation was enhanced, suggesting an additional pathway of TMA-GPSA-induced complement activation when antibody is present.

Activation of the complement system in the BAL was coincident with the increase in protein and RBC observed in the BAL after TMA-GPSA challenge but preceded the influx of inflammatory cells into the airway and lung tissue. These observations are consistent with a role for complement activation products in TMA-induced hemorrhage and infiltration of eosinophils and macrophages.

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**Fig. 7.** Lung edema expressed as the wet/dry weight of lung from nonsensitized and 0.3% TMA-sensitized animals 24 h after intratracheal instillation of GPSA or TMA-GPSA. *p < 0.05; ns, not significant.
In our studies, we found that C3a concentrations increased significantly in BAL but not in plasma after TMA-GPSA challenge. A small but significant decrease in plasma C3a was observed in 0.3% TMA-sensitized animals challenged with TMA-GPSA, but not in nonsensitized or 30% TMA-sensitized animals. However, the decrease in C3a in the 0.3% TMA-sensitized animals was also observed in the absence of antigen (GPSA-instilled control animals). The data therefore suggest that the decrease in C3a observed in plasma is not due to interaction of antigen and antibody and thus not dependent on sensitization. No correlation between the increased C3a concentrations in the BAL and the decrease in plasma C3a concentrations measured before and after challenge was detected, suggesting that the increase in BAL C3a was not due to movement of C3a from the plasma to the BAL. Also, the hematocrit before and after TMA-GPSA challenge was not significantly different, indicating that large changes in plasma volume were not occurring after TMA-GPSA challenge. These data suggest that TMA-GPSA activates the complement system locally in the airspace. However, it is possible that C3a generated in the circulation moved into the airspace with the lung edema and hemorrhage. Reports in the literature vary as to whether complement activation occurs in asthmatics, either systemically or in the airspace (Regal, 1997b). Measuring C3a in the plasma and BAL, van de Graaf et al. (1992) suggested that C3a was generated locally in the lung in a subset of asthmatics. Complement proteins are known to be synthesized in the liver, and recent studies by Yasojima et al. (1998, 1999) have demonstrated synthesis of complement proteins in the heart and brain. Previous research has shown that synthesis of complement proteins may also occur in the lung and be independent of complement synthesis in the liver (Pennington et al., 1979; Alpert et al., 1984). Alveolar macrophages, alveolar type II epithelial cells, and lung fibroblasts are known to synthesize proteins of both the classical and alternative complement pathways (Perlmutter et al., 1991). Also, both pathways of complement activation have been shown to be functionally intact (through C5) in BAL obtained from rabbits (Henson et al., 1979; Giclas et al., 1987). In our previous studies, the amount of C3 in the BAL increased after TMA-GPSA challenge (Fraser et al., 1995), suggesting that TMA-GPSA may induce the synthesis of complement proteins. Alternatively, significant quantities of C3 from the circulation may have entered the airways after TMA-GPSA challenge because of increased microvascular permeability and hemorrhage.

Occupational exposure to TMA for weeks to years is thought to be required before TMA elicits asthma (Hagmar et al., 1987). However, the lack of a clear association between antibody and TMA-induced asthma, as well as reports that low molecular weight chemicals can cause asthma without sensitization (Chan-Yeung and Malo, 1994), raised the question of the necessity of sensitization in TMA-induced asthma. In our studies, GPSA control and TMA-GPSA challenge were compared within each sensitization group, and this analysis indicated that TMA-GPSA challenge elicited cellular infiltration only in sensitized animals. However, when we tested for differences in the magnitude of the GPSA/TMA-GPSA effect between sensitization groups, the analysis indicated that an increase in the number of eosinophils in the BAL and lung was clearly dependent upon sensitization. Significant interactions of the GPSA/TMA-GPSA effect with sensitization were not detected for the other variables. The data therefore suggest that in the absence of sensitization, TMA-GPSA induces increases in C3a concentrations, numbers of neutrophils, macrophages, and RBC, and total protein in the airspace and numbers of neutrophils in the lung. In contrast, eosinophils in the airspace and lung do not increase after TMA-GPSA challenge in nonsensitized animals. TMA-GPSA-induced eosinophilia occurs only after sensitization.

An effect of sensitization on the number of resident inflammatory cells was also demonstrated. Sensitization to TMA affected the number of eosinophils in the BAL as well as the numbers of eosinophils and neutrophils in the lung tissue in GPSA control animals. This change in the number of cells residing in the lung due to sensitization may affect not only the response that occurs upon TMA challenge but also the response due to other insults. Sensitization with either 0.3% TMA or 30% TMA was intended to create differing magnitudes of response upon challenge. TMA-specific IgG antibody was significantly greater in guinea pigs sensitized with 30% TMA than those sensitized with 0.3% TMA (C. P. Larsen and J. F. Regal, unpublished data). However, the extent of inflammatory cell infiltration after TMA-GPSA challenge was not significantly different between 0.3 and 30% TMA-sensitized animals for all variables, suggesting sufficient antibody was produced with the 0.3% protocol to react with the dose of TMA-GPSA used for challenge.

Limited studies were also conducted to determine the effect of intratracheal instillation itself on the cells in the lung. Our studies indicated that GPSA instillation caused increases in the numbers of macrophages and red blood cells in the BAL as well as a decrease in lung EPO activity. As might be expected, instillation of the protein GPSA had minor but significant effects on the responses measured. These data verify the need to make all comparisons of TMA-GPSA instillation with a control GPSA instillation of equivalent amounts of protein.

The magnitude of the TMA-GPSA-induced response in sensitized guinea pigs correlates with the extent of complement activation. In sensitized animals C3a concentrations in the BAL correlate with all of the responses measured, except neutrophils in the lung. The fact that C3a correlated with numbers of eosinophils, macrophages, and RBC in the BAL of sensitized animals supports our previous work with complement-depleted animals (Fraser et al., 1995), suggesting that complement plays an important role in mediating the TMA-GPSA-induced increase in these variables. A significant TMA-GPSA-induced increase in C3a also occurred in nonsensitized guinea pigs. However, in nonsensitized animals, none of the responses measured significantly correlated with the concentration of C3a except for total protein in the BAL. The data from this study and our previous work (Fraser et al., 1995) suggest that complement activation is important in the TMA-GPSA-induced response in sensitized animals.

Previous studies had not determined whether TMA-GPSA instillation caused significant lung edema in sensitized or nonsensitized guinea pigs. Thus, we determined the effect of TMA-GPSA instillation on the wet/dry ratio of the lung. The wet/dry weight of the lung was similar in both nonsensitized and sensitized animals after TMA-GPSA challenge. However, when GPSA control and TMA-GPSA-challenged animals within each sensitization group were compared, the TMA-GPSA-induced change was only significant in sensi-
tized animals. Using one-way ANOVA, the wet/dry ratio of the lung in GPSA-challenged animals was not different in nonsensitized versus sensitized animals. When we tested for the effect of sensitization on the magnitude of the GPSA versus TMA-GPSA effect (Table 1), no significant difference was observed. Thus, the data overall suggest that lung edema following TMA-GPSA instillation is independent of antibody. This conclusion is similar to the findings with the other responses measured in our study, with the exception of the eosinophilia.

We have demonstrated that the complement system is activated in response to TMA-GPSA not only in the presence of specific antibody but also in the absence of antibody. Both antibody-dependent and -independent mechanisms of TMA-GPSA-induced complement activation appear to exist. Complement proteins play a role in the influx of cells into the airspace after TMA-GPSA challenge, but complement activation is not sufficient for the complete response to occur. In particular, eosinophilia in the lung requires both sensitization and complement for full expression.

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References


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