

# Immune responses of cynomolgus monkeys to phthalic anhydride

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Four groups of four *Macaca fascicularis* monkeys were administered 10 consecutive weekly subcutaneous injections of 2 mg aluminum hydroxide plus one of the following: 200 µg of phthalic anhydride (PA)-monkey serum albumin (PA-MSA, group 1); 200 µg of PA dissolved in ethanol-saline (EtOH-sal, group 2); 200 µg of MSA (group 3); or EtOH-sal alone (group 4). Direct intracutaneous tests to PA-MSA, PA-EtOH-sal, MSA, and EtOH-sal were applied at biweekly intervals throughout the course of the immunization. Serum-specific IgG to PA-MSA and specific IgE to PA-MSA were determined at 2-week intervals according to the ELISA and RAST methods, respectively. The prevalence of cutaneous sensitivity to PA-MSA in the PA-MSA-immunized group (group 1) was significantly greater after 4 and 6 ( $p < 0.01$ ) and 8 and 10 ( $p < 0.05$ ) weeks, compared with the other treatment groups. Significantly elevated ( $p < 0.01$ ) PA-MSA-specific IgG was also observed in monkeys in group 1 compared with the other treatment groups. No significant changes in PA-MSA RAST or total IgE were observed in any group during the study. These results indicate that parenteral sensitization to PA in subhuman primates requires the presence of new antigenic determinants formed by PA on protein carriers. (*J ALLERGY CLIN IMMUNOL* 1988;82:23-9.)

PA (benzene orthodicarboxylic acid anhydride; molecular weight 148.12 gm) is a white lustrous compound that is slightly soluble in water and soluble in alcohol and ether. It is a commercially important reagent widely used in the plastics industry as a curing agent for epoxy resins, as a plasticizer for vinyl chloride polymers, in the production of unsaturated polyesters (PA is used in conjunction with maleic anhydride to promote hardness in the final product for use in boats and automotive and recreational vehicles),

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## Abbreviations used

|                                 |   |
|---------------------------------|---|
| PA:                             | Phthalic anhydride                                |
| P-K:                            | Prausnitz-Küstner reaction                        |
| NAD:                            | New antigenic determinant                         |
| MSA:                            | Monkey serum albumin                              |
| Alum:                           | Aluminum hydroxide                                |
| PA-MSA:                         | Phthalic anhydride-monkey serum albumin conjugate |
| EtOH-sal:                       | Ethanol saline                                    |
| PBS:                            | Phosphate-buffered saline solution                |
| V <sub>2</sub> O <sub>5</sub> : | Vanadium pentoxide                                |
| K:                              | Rate constant                                     |
| ln:                             | Natural logarithm                                 |
| a:                              | Original concentration                            |
| x:                              | Concentration at any time                         |
| t:                              | Time  |
| t <sub>1/2</sub> :              | Half-life   |
| BSA:                            | Bovine serum albumin                              |

and in alkyd resins (paints and coatings). It is also used in the production of dye intermediates. Occupational exposures to PA dust occur during the loading of the solid powder into bags and during their subsequent unloading. In addition, occupational exposures to vapors and fumes of PA may occur when it is heated.<sup>1</sup>

**TABLE I.** Previous experimental history of study animals

| Group 1 | Group 2 | Group 3 | Group 4 |
|---------|---------|---------|---------|
| B23*†   | B24*‡   | B32*†‡  | B31*‡   |
| B39*†   | B42*    | B44*    | B43*‡   |
| B45*‡   | C46*    | C55     | C52*    |
| C56     | C62*    | C83*    | C72     |

\*Monkeys were previously challenged (from 4 to 44 months before the present study) diagnostically by bronchoprovocation with an aqueous extract of cotton (1-minute exposure).

†Monkeys were previously injected (12 months before the present study) parenterally with small amounts of V<sub>2</sub>O<sub>5</sub>-protein conjugate.

‡Monkeys were previously challenged (24 months before the present study) diagnostically by bronchoprovocation by inhalation of V<sub>2</sub>O<sub>5</sub> dust.

Inhalation of PA dust or vapor produces a spectrum of respiratory effects in occupationally exposed workers. Irritating effects are generally experienced by most workers after an acute exposure to high concentrations of dust or vapors. Coughing, sneezing, and a burning sensation in the nose and throat are reported after inhalation. Irritation of unprotected eyes and skin can occur after PA exposure.

Clinical evaluations of workers exposed to PA have been conducted by several investigators.<sup>1-9</sup> An allergic pathogenesis for PA-induced asthma was suggested because clinical sensitization develops after a latency period of at least several months in a small number of exposed workers. Evidence for circulating reaginic antibodies was first described in 1939 by Kern,<sup>4</sup> who demonstrated positive P-K and positive skin test responses to PA in a chemist with occupational asthma. It was not until 1976 that Maccia et al.<sup>5</sup> demonstrated the presence of specific IgE antibody against PA-protein conjugate by the RAST method. Subsequent investigations of acid anhydride-sensitized workers have demonstrated that the reaginic response is directed against NADs formed by interaction of these reactive small molecular weight compounds with selected protein carrier molecules.<sup>8</sup> Antibodies to these NADs exhibit variable specificity and cross-reactivity.<sup>9</sup> Although they are present in all sensitized workers, it has not yet been determined whether these allergic determinants are a prerequisite for allergic sensitization or whether NADs can be formed spontaneously in vivo after significant exposure to the chemical hapten alone.

The purpose of the present study was to investigate the immunologic conditions required for sensitization to PA alone or PA complexed with a protein carrier (MSA) in cynomolgus monkeys. Cynomolgus mon-

keys were used in this investigation because of their previously documented usefulness in the study of occupational allergens/haptens<sup>10-14</sup> and their phylogenetic, immunologic, and physiologic similarities to humans.

## METHODS AND MATERIAL

### Study design

The four groups of four animals received weekly subcutaneous inguinal injections (0.2 ml) containing 2 mg of alum<sup>15</sup> and one of the following: group 1, 200 µg of PA-MSA conjugate containing  $4.7 \times 10^{-8}$  mol PA and group 2,  $1.4 \times 10^{-6}$  mol PA (100.0% pure, lot 124142, obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.). The PA was solubilized in EtOH-sal (first solubilized in 0.5 ml of ethanol and then diluted in 9.5 ml of 0.02 mol/L phosphate buffer, pH 7.4, containing 0.9% NaCl PBS); group 3, 200 µg of MSA (chromatographically purified cynomolgus MSA, lot 23844, Cappel Worthington, Cooper Biomedical, Malvern, Pa.); and group 4, EtOH-sal alone. The animals were also tested intracutaneously (100 µl) biweekly with histamine (0.1% histamine diphosphate, Eli Lilly, Indianapolis, Ind.); EtOH-sal, PBS, and MSA (10 mg/ml); and serial tenfold dilutions of  $10^{-3}$  to  $10^{-7}$  mol/L PA and  $10^{-6}$  to  $10^{-10}$  mol/L PA-MSA (based on PA content). All solutions were made fresh within ¾ hour before use.

### Animals

Sixteen young adult male cynomolgus monkeys (*Macaca fascicularis*, Primate Imports Corporation, Port Washington, N.Y.) were selected for use in this study. All were in excellent health. They had been maintained in the National Institute for Occupational Safety and Health primate colony and had no previous experimental exposure to PA. The animals had undergone routine (3-month intervals) evaluation for tuberculosis (intradermal tests on upper eyelid with 0.1 ml of tuberculin, mammalian human isolates, Jensen-Salsbery Laboratories, Division of Burroughs Wellcome Co., Kansas City, Mo.) and parasites (fecal examination) with uniformly negative results. In addition, during a 3.66-year period before the present investigation, some of the animals had been challenged diagnostically by bronchoprovocation with an aqueous extract of cotton dust or V<sub>2</sub>O<sub>5</sub> dust or injected with V<sub>2</sub>O<sub>5</sub>-protein conjugates (Table I). These animals were included in the design of the present experiment because of evidence for reversibility of similar cotton diagnostic challenges in humans,<sup>6</sup> absence of detectable changes in pulmonary function after V<sub>2</sub>O<sub>5</sub> dust challenges, and the lack of positive vanadium skin test results in the animals injected with V<sub>2</sub>O<sub>5</sub>-protein conjugates. In addition, baseline immunoglobulin levels (before the start of the present experiment) were within normal ranges<sup>11</sup> for healthy cynomolgus monkeys. Maximal use of these valuable research resources by repeated experimentation (when these were believed not to compromise the outcome of subsequent experiments) is consistent with the Public Health Service Policy on Humane Care and Use of Laboratory Animals<sup>17</sup> and the Health Research Extension Act of 1985 (Public Law 99-158).

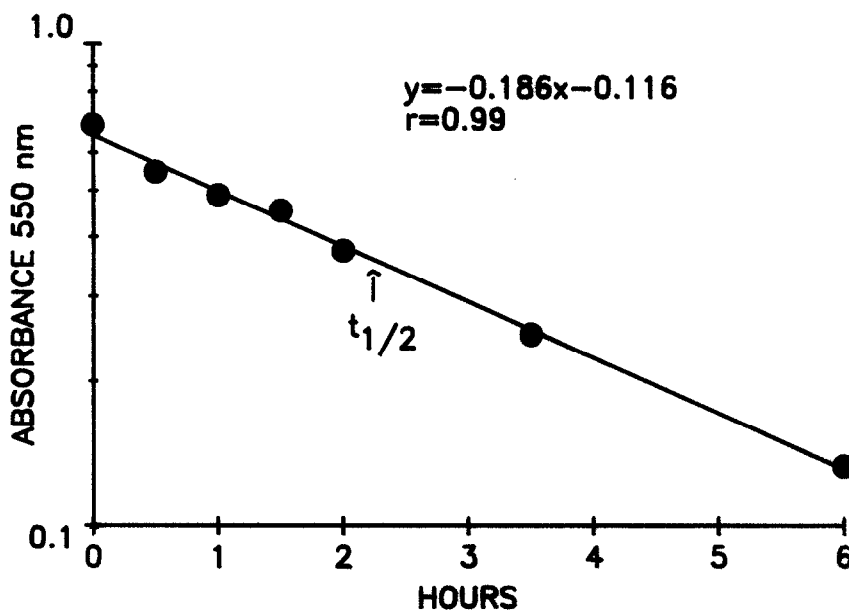


FIG. 1. Semilogarithmic plot of absorbance at 550 nm versus time in hours for the reaction of PA with ethanol. The best-fit simple orthogonal regression line is illustrated ( $r = 0.99$ ;  $p < 0.0001$ ).

The animals' weights ranged from 4.5 to 6.0 kg. The monkeys were fed standard chow, daily (Monkey Chow Jumbo, Ralston Purina Co., St. Louis, Mo.), and fresh fruit (apples and bananas), three times weekly, and maintained on a 12-hour photoperiod (lights on, 7 AM; lights off, 7 PM). Water was provided ad libitum.

The animals were randomly assigned to four treatment groups ( $n = 4$ ) as outlined in Table I.

### PA analysis

EtOH-sal was used to solubilize PA because of its low solubility in physiologic solutions. To determine its stability in ethanolic solutions, PA was solubilized in ethanol (6.75 mg/ml) and allowed to stand at room temperature for 22 hours. Aliquots were taken at 0-, 1/2-, 1-, 1 1/2-, 2-, 3 1/2-, 6-, and 22-hour intervals for anhydride analysis. The anhydride content of the 0.37 ml aliquots, diluted in 10 ml of acetonitrile, was estimated by reaction with 2-nitrophenylhydrazine (Aldrich Chemical Co., Milwaukee, Wis.).<sup>18</sup> A concurrent control of PA in acetonitrile was also assayed. Blue hydrazone products formed were analyzed by ultraviolet spectroscopy at 550 nm (Beckman DU-6, Beckman Instruments, Inc., Fullerton, Calif.). All determinations were performed in duplicate. The amount of anhydride present at each sampling period was calculated by the following formula to determine K:  $\ln[a/(a - x)] = Kt$ . The  $t_{1/2}$  for anhydride reaction was determined by  $t_{1/2} = 1.386/K$ .

### Tranquilization and sera collection

The monkeys were tranquilized by injection (intramuscularly) with a mixture of 70 mg/ml of ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, N. Y.) and

6 mg/ml of xylazine (Rompun; Bayvet Division of Cutter Laboratories, Shawnee, Kan.) at 0.15 ml/kg body weight.<sup>19</sup> Blood was withdrawn biweekly for sera collection by femoral venipuncture. The blood was allowed to clot at room temperature, and the sera were separated by centrifugation at 1000 g for 10 minutes. The sera were stored at  $-20^{\circ}\text{C}$  until use.

### Intracutaneous testing

Skin testing was performed in tranquilized animals before and every 2 weeks during the study.<sup>11</sup> Areas of the chest and thorax were shaved, and 5 ml of a 0.5% solution of Evans blue dye was injected intravenously (saphenous vein). Fifteen minutes later 100  $\mu\text{l}$  aliquots of the test reagents were injected intracutaneously into the animals. The injection sites were observed at 30 minutes for cutaneous bluing reactions. A circular millimeter template was used to measure the largest diameters of positive skin reactions.

### Preparation of PA-MSA conjugates

PA-MSA conjugates were prepared according to the method of Bernstein et al.<sup>8</sup> The number of moles of PA conjugated to the protein carrier was calculated by determining the difference in optical density (at 240 and 280 nm) between conjugated and unconjugated PA-MSA.<sup>7</sup> The final PA-protein conjugate had a concentration of 10.0 mg of protein per milliliter and  $23.5 \times 10^{-8}$   $\mu\text{mol}$  of PA per milliliter (235  $\mu\text{mol/L}$  with respect to PA).

### Determination of total IgE and specific IgG and IgE to PA-MSA

The ELISA method used to measure PA-MSA-specific IgG was the indirect method described previously by Voller

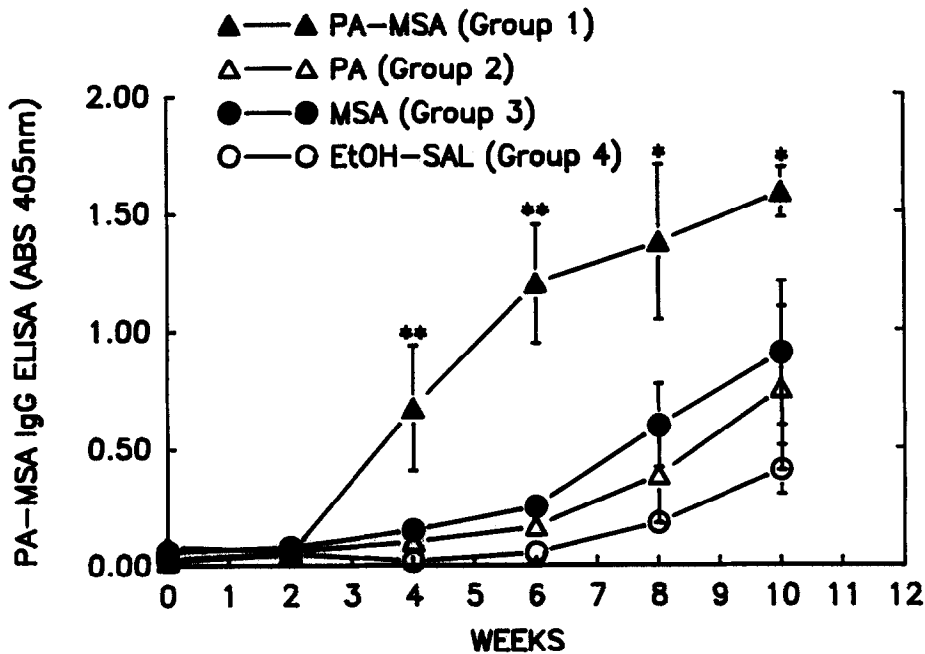


FIG. 2. PA-specific IgG (ELISA; serum dilution 1:90) ( $n =$  four monkeys per group). Data are presented as mean  $\pm$  SEM; sera were obtained at 2-week intervals (x axis); treatments were performed weekly. \* $p < 0.05$ ; \*\* $p < 0.01$ , Kruskal-Wallis analysis of variance.

et al.<sup>20</sup> Aliquots of 0.15 ml of PA-MSA at a concentration of 180  $\mu\text{g/ml}$  diluted in 0.1 mol/L  $\text{NaHCO}_3$  (pH 8.6) were placed in each well and incubated at room temperature for 2 hours and 18 at 4 $^\circ\text{C}$  in flex micro-ELISA U-plates. Each well was then washed three times with PBS, and a similar wash was repeated between all subsequent steps. Next, 0.2 ml samples of each experimental monkey serum at three different dilutions (1:9, 1:45, and 1:90 in 5% BSA) were added and incubated for 2 hours at room temperature. To measure specific binding of IgG, 0.15 ml of goat antihuman IgG alkaline phosphate conjugate (Sigma Chemical Co., St. Louis, Mo.; diluted 1/100 in 1% BSA) was added to the wells for 2 hours at room temperature. Finally, 0.15 ml of 0.0006 mol/L of p-nitrophenyl phosphate disodium (Sigma Chemical Co., St. Louis, Mo.) substrate solution, diluted in alkaline glycine buffer (0.05 mol/L glycine and 0.5 mmol/L magnesium chloride; pH 10.4), was added. After 10 minutes the reactions were terminated with 0.05 ml of 2 mol/L NaOH. Optical density at 405 nm was read on a micro-ELISA MR590 spectrophotometer (Dynatech Laboratories, Inc., Alexandria, Va.).

RAST testing for PA-specific IgE antibodies was performed with PA-MSA coupled to methylcellulose disks by cyanogen bromide treatment, and the RAST analysis was performed as described previously.<sup>21</sup> For all PA-specific in vitro antibody evaluations with monkey sera, a positive control of serum from a PA-sensitive worker was run simultaneously. Total serum IgE levels were determined by radioimmunoassay (Quantitope, a polyclonal radioimmunoassay kit, Kallestad Laboratories, Austin, Texas).

### Statistical analyses

Fisher's exact test (Epistat Statistical Package, Dr. Tracy Gustafson, Round Rock, Texas) was used to investigate the prevalence of positive PA-MSA skin test conversions (positive test results at  $10^{-6}$  mol/L or less PA-MSA, based on the PA content of PA-MSA). Simple orthogonal regression was used to investigate the linearity of the reaction of PA in ethanol. All other hypothesis tests were performed by nonparametric methods (Number Cruncher Statistical System, Dr. Jerry Hintze, Kaysville, Utah). Kruskal-Wallis analysis of variance and Mann-Whitney U tests were used to determine if there was a change in immunoglobulin levels at any biweekly testing period among the groups or across all biweekly testing periods within groups. A type 1 error level of  $p < 0.05$  was considered statistically significant for analysis of variance results. To adjust for multiple comparisons, the minimum  $p$  value possible for each Mann-Whitney U test ( $p < 0.025$ ) was considered statistically significant.

### RESULTS

The absorbance of nitrophenylhydrazine reaction products from aged PA solutions in ethanol decreased over time, indicating that the anhydride had reacted, presumably forming ethyl esters with ethanol. No anhydride was depleted in acetonitrile solvent used for this analytic assay after 22 hours. A semilogarithmic plot of absorbance versus time for the reaction yielded a highly significant linear relationship ( $r = 0.99$ ;

**TABLE II.** PA-MSA conjugate skin test conversions in monkeys treated weekly with PA-MSA (group 1)\*

| Animal identification | Week              |                   |                  |                   |                   |                   |
|-----------------------|-------------------|-------------------|------------------|-------------------|-------------------|-------------------|
|                       | 0                 | 2                 | 4                | 6                 | 8†                | 10†               |
| B23                   | >10 <sup>-6</sup> | >10 <sup>-6</sup> | 10 <sup>-7</sup> | >10 <sup>-6</sup> | >10 <sup>-6</sup> | >10 <sup>-6</sup> |
| B39                   | >10 <sup>-6</sup> | 10 <sup>-6</sup>  | 10 <sup>-7</sup> | 10 <sup>-6</sup>  | 10 <sup>-6</sup>  | 10 <sup>-6</sup>  |
| B45                   | >10 <sup>-6</sup> | NT                | NT               | 10 <sup>-6</sup>  | 10 <sup>-6</sup>  | 10 <sup>-6</sup>  |
| C56                   | >10 <sup>-6</sup> | >10 <sup>-6</sup> | 10 <sup>-6</sup> | >10 <sup>-6</sup> | 10 <sup>-6</sup>  | 10 <sup>-6</sup>  |

NT = not tested.

\*Data are expressed as lowest PA-MSA concentration (10<sup>-x</sup> mol/L, based on PA content of PA-MSA) yielding positive bluing reactions. If no positive reaction was observed at 10<sup>-6</sup> mol/L PA-MSA, the data are presented as >10<sup>-6</sup> mol/L. Positive reactions yielded bluing diameters ranging from 7 to 12 mm.

†*p* < 0.01. Fisher's exact test.

*p* < 0.001), indicating that the reaction follows a first-order kinetic profile (Fig. 1). The calculated rate constant for the reaction was 0.32 ± 0.02 (mean ± SEM). This value elicits a *t*<sub>1/2</sub> for reaction of PA in ethanol of 2.16 ± 0.13 hours (mean ± SEM).

After 4 and 6 weeks of injections, the group injected with PA-MSA (group 1) had significantly (*p* < 0.01) increased levels of PA-MSA-specific IgG compared with the other experimental groups (Fig. 2). After 8 and 10 weeks of experimental PA-MSA treatment, this same group of animals still had elevated PA-MSA-specific IgG, however at a lessened level of statistical significance (*p* < 0.05).

There were significant increases over baseline values for PA-MSA-specific IgG in all treatment groups compared with their respective baseline values. The EtOH-sal (group 4) and PA (group 2) treatment groups had statistically significant (*p* < 0.025) increases in PA-MSA-specific IgG at the 10-week testing period, whereas the MSA (group 3) treatment group had elevated values (*p* < 0.025) after eight weekly injections. The PA-MSA treatment group (group 1) had significantly elevated PA-MSA-specific IgG values, compared with their baseline, after 4, 6, 8, and 10 weeks of treatment (all *p* < 0.025). No significant changes over time were detected for levels of the other immunoglobulins measured. PA-MSA injections yielded positive immediate skin test conversions in the PA-MSA exposure group only (group 1) compared with the other treatment groups. At baseline all animals of all groups had negative skin test results at 10<sup>-6</sup> mol/L PA-MSA. The prevalence of positive PA-MSA skin test results in the PA-MSA exposure group (group 1) was significantly greater (*p* < 0.01) compared with the other treatment groups after eight and 10 weekly injections. Skin test changes observed

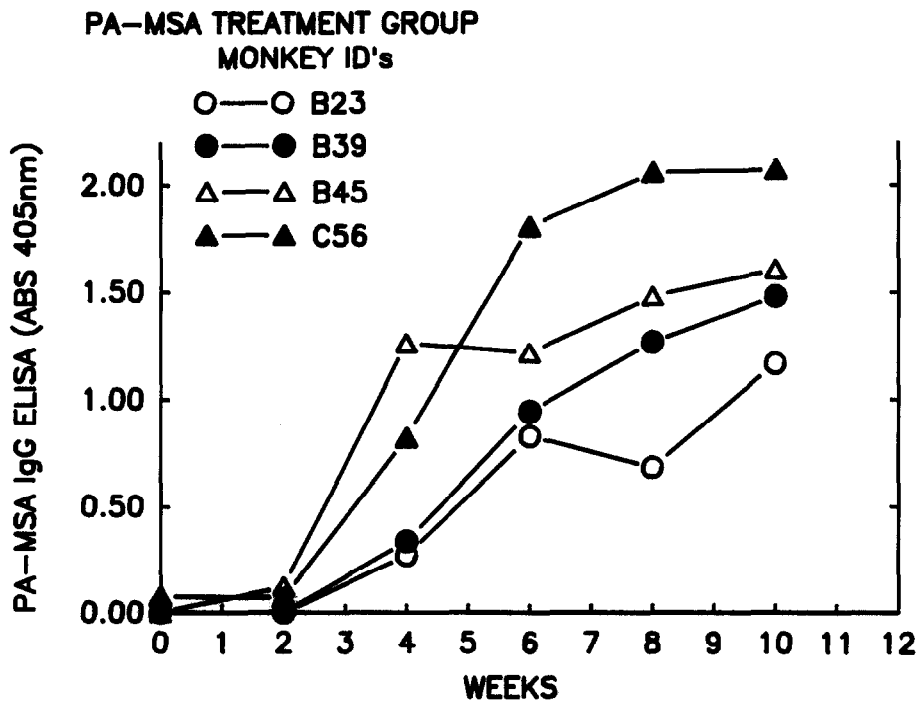
in animals in group 1 are outlined in Table II. Positive control histamine skin test results for all monkeys for all testing periods elicited a mean bluing diameter of 17.5 ± 3.5 mm. No positive PA or PA-MSA skin tests were observed in any of the other groups at any of the testing periods.

No statistically significant changes in PA-MSA RAST results were observed for any group at any of the biweekly sera acquisition periods. In addition, no individual animals at any testing period had RAST binding activity >2 SD above the mean of baseline RAST results.

Total IgE levels for the four groups of monkeys were not significantly different either from baseline or at any 2-week testing period.

## DISCUSSION

The present study demonstrates that weekly injections of PA-MSA conjugate (with alum) in monkeys significantly increase PA-MSA-specific IgG antibody levels and produce positive skin test results to high dilutions of PA-MSA. There appears to be a waning of skin test sensitivity that is inversely correlated to the increasing PA-MSA-specific IgG levels. Bi-weekly skin testing with PA, PA-MSA, EtOH-sal, and MSA was effective in increasing PA-MSA-specific IgG values in the groups not parenterally administered PA-MSA conjugate compared with each group's respective baseline. It is interesting to note that the group treated biweekly with PA-MSA, in addition to skin testing, demonstrated significant elevations in PA-MSA-specific IgG (compared with the other treatment groups), after four weekly treatments, 4 to 6 weeks before the observation of elevated PA-MSA-specific IgG in the other treatment groups. These results indicate that skin testing alone (presumably from



**FIG. 3.** PA-specific IgG (ELISA; serum dilution 1:90) of individual monkeys of PA-MSA treatment group. Sera were obtained at 2-week intervals (x axis); treatment was performed weekly.

PA-MSA testing solutions) without parenteral injection of PA-MSA is moderately effective in elevating circulating titers of PA-MSA-specific IgG. In addition to skin testing, parenteral weekly injections of PA-MSA offer significantly greater stimulus to the production of PA-MSA-specific IgG compared with the other treatments. Total IgE and PA-specific IgE were not significantly affected from the experimental protocols of the present study.

The  $t_{1/2}$  for stability of PA solutions in ethanol was demonstrated to be  $>2$  hours, indicating that the freshly prepared solutions used for skin testing and immunization were essentially in the anhydride form when they were administered.

The findings of this study agree with one of the theoretic variants of the immunopathogenesis of acid anhydride occupational asthma, as outlined by Bardana et al.,<sup>22</sup> who described large amounts of measurable PA-specific IgG versus small or immeasurable amounts of IgE-specific antibody. These findings also agree with the recent work of Nielsen et al.,<sup>1</sup> who studied 28 workers exposed to PA and found PA-specific IgE and a positive PA-human serum albumin skin test result in one individual, whereas PA-specific IgG was found in four workers, two of whom were considered asthmatic. In another recent study, Nordman et al.<sup>23</sup> found that elevated PA-HSA IgG<sub>4</sub>

levels were almost invariably associated with work-related symptoms (rhinitis, breathlessness, or conjunctival irritation) from PA exposure. In the present study evidence for both reaginic and IgG immune responses was demonstrated in monkeys injected with PA-MSA. The induction of marked PA-MSA dermal sensitivity in some of the monkeys suggests that reaginic mechanisms may have been present. Although there was no statistically significant evidence for PA-MSA-specific IgE found in the monkeys, this may have been due to the demonstrated reduced sensitivity of RAST testing with monkey sera with human reagents.<sup>11</sup> In addition, it has been demonstrated that heterogeneous immunologic responses (including specific IgE and IgG) are elicited in humans exposed to acid anhydride.<sup>10</sup> Interference (blocking antibody) between these antibody classes may also have lowered the sensitivity of the RAST assay. Asthmatic symptoms have been reported to disappear in certain exposed individuals despite continuing PA exposure.<sup>6</sup> This again may be related to the induction of blocking IgG antibody classes from PA exposure that could be protective. The apparent decrease in PA-MSA skin sensitivity found in some of the monkeys of the present experiment agrees with reduction in skin sensitivity on continued PA exposure reported in humans. It is possible that elicitation of significant

PA-MSA-specific IgG could act as blocking antibody that could have modified the PA-MSA-specific IgE responses.

It appears that the conditions for maximal production of antibody from PA exposure in monkeys requires either prior sensitization to hapten-dependent determinants on a carrier protein (MSA in this case) or prior sensitization of hapten-independent new antigenic determinants from exposure to the reactive phthalate group compounds. Workers who are sensitized to PA occupationally most probably form hapten conjugates with proteins present in the airway or airway epithelium. The lack of cutaneous sensitization to PA or PA-MSA in the group of immunized monkeys exposed to PA alone supports this hypothesis.

In conclusion, results of the present experiment indicate that PA-MSA injections in monkeys are effective in inducing positive skin sensitivity and statistically significant de novo synthesis of specific IgG to PA-MSA.

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