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Acute autoimmune response in a case of pyromellitic acid dianhydride-induced hemorrhagic alveolitis

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A 17-year-old man was occupationally exposed to pyromellitic acid dianhydride dust during the production of epoxy resin in a chemical factory. He was clinically diagnosed as having acute hemorrhagic alveolitis associated with anemia. The serologic analysis revealed a high concentration of IgG antibodies against pyromellitic acid dianhydride-treated human serum albumin (PMDA-HSA). Immunoblotting with PMDA-treated human serum as antigen and the patient's serum as the first antibody showed that additional PMDA-modified serum proteins other than HSA were recognized by the patient's IgG antibodies in the higher mol. mass range (> 67 kDa). No specific IgG could be detected against other anhydride conjugates (maleic acid, MA; phthalic acid, PA) with the exception of a reaction with the trimellitic acid anhydride-conjugated HSA (TMA-HSA). No specific IgE antibodies could be detected against any of the above mentioned antigens, but immunoblotting of the patient's serum indicated IgG4-type autoantibodies against *in vitro* PMDA-treated Ig molecules of normal serum proteins.

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The acid anhydrides are a group of low mol. mass chemicals used mainly in the production of plastics, epoxy resins, printing dyes, adhesives, and paints. In water, they hydrolyze quickly to the corresponding nonreactive acids, while in the anhydride state they are very reactive, mainly with the amine and hydroxyl groups. Acid anhydrides have been reported to induce immunologically mediated asthma in exposed workers (6, 7), and specific IgE responses in man have been attributed to HSA modified by phthalic (PA), trimellitic (TMA), tetrachlorophthalic, hemic, hexahydrophthalic, and maleic acid anhydrides (MA) (6). The specificity studies on the IgE antibodies detected in the serum of workers sensitized to TMA indicate that the antibodies were directed against a TMA-HSA conjugate, and it was postulated that the reaction of TMA with albumin altered the albumin molecule to form new antigenic determinants (7). The underlying pathomechanism of the reaction of the dust of acidic anhydrides with serum proteins upon inhalation remains to be elucidated. Topping et al. have tested antibody specificity by RAST inhibition assays from different sensitized subjects, and found that in some subjects the IgE was specific for new antigenic determinants, whereas in others the antibody showed hapten specificity (5).

This is the first report of a pyromellitic acid dianhydride (PMDA)-induced sensitization. PMDA is increasingly used in the chemical industry as a cross-linking agent, and the present annual production in Germany exceeds 1000 metric tons. A workplace exposure in the production and application of PMDA seems to be inevitable.

In the present work we investigated the antibody specificities of a patient exposed to PMDA dust in a chemical plant who was suffering from acute hemorrhagic alveolitis.

Material and methods

Patient

A 17-year-old, otherwise healthy, nonsmoker chemical industry worker (S.M.) was directly involved in the production of epoxy resin in a Swiss chemical plant for 2 years. The resin consists of LY1025 (Biphenol A, Ciba Geigy), diluent DY026SP (1–4 butandiol-diglycidyl ether, Ciba Geigy), and zinc stearate. PMDA serves as the cross-linking agent and is added to the above mixture in large quanti-

ties in powder form. It was the responsibility of S.M. to prepare the resin mixture. As result of acute symptoms, including fever, chills, and chest discomfort, S.M. was suddenly admitted to the emergency ward (3). He complained of headache, nausea, and retrosternal pain.

He had no cough and he did not produce any sputum. He was pale and the body temperature was 38.2 °C. The pulse rate was 130 beats/min. Crackles could be heard over both lungs. The erythrocyte sedimentation rate was 18 mm/h, the hemoglobin level was 11.5 g/dl, the mean corpuscular volume was 87 fl, the reticulocyte count was 1%, and the leukocyte count was 24000/µl. Bilirubin and lactic dehydrogenase levels were normal. Coombs' test was negative. No clothing abnormalities were detected. Results of the urinalysis were normal. The chest radiograph showed bilateral acinar opacities. The pulmonary function test showed a slight restrictive defect: vital capacity, 3.740 ml (78%); FEV_1, 3.260 ml (97%); FEV_1/vital capacity ratio, 0.87. Carbon monoxide diffusing capacity (Krogh factor) was increased to 228%.

Clinical examination revealed an acute hemorrhagic alveolitis with anemia. The anti-double-strand DNA antibody concentration was slightly elevated (44 IU/ml), and the IgG anticardiolipin antibody titer was highly positive.

The patient was treated with 1 g methylprednisolone and 150 mg cyclophosphamide four times a day for 4 d.

For the immunologic analysis, blood was taken without anticoagulants, sent to our laboratory (Ruhr-University of Bochum, Germany), and centrifuged. The serum was aliquoted and kept at −20 °C until use. A serum pool from 10 occupationally unexposed subjects was treated in the same way and used as negative control.

Reagents

HSA, all antisera, and *p*-nitrophenyl-phosphate (PNP) substrate were obtained from Sigma Chemical Co (Munich, Germany). High-purity-grade anhydrides were purchased from Merck (Darmstadt, Germany).

Preparation of anhydride-HSA conjugates

PMDA-HSA, TMA-HSA, PA, and MA-HSA conjugates were prepared as described by Zeiss (7), except that the calculated molar ratio of TMA- and PMDA-HSA of 300:1 was used. The mixture was then ultrafiltrated with an Amicon filtration unit with a molecular cutoff limit of 25 kDa. The actual molar ratios of anhydrides to HSA after conjugation were not calculated.

Preparation of PMDA human serum conjugates

The human serum conjugate was prepared in a similar manner, except that the serum was prediluted to 1:32 with PBS before the anhydride treatment.

Allergen disks

Paper disks (Schleicher and Schüll) were activated with CNBr, and the anhydride HSA conjugates were immobilized on the paper disks in 0.1 mol/l sodium bicarbonate, pH 9.5, at a concentration of 1 mg protein per ml. The disks were then sorted at 4 °C until use. Purified HSA was also coupled to paper disks at the same protein concentration to serve as a negative control.

Enzyme-allergosorbent test (EAST)

EAST was done by incubating 50 µl of the patient's serum or serial dilutions of 50 µl of the serum with the allergen disks for 180 min at room temperature. After that, the disks were washed three times with 2 ml PBS containing 0.1% Tween-20 and incubated again with 50 µl monoclonal murine antihuman IgG or IgE (diluted to 1:5000 in PBS) overnight. This was followed by repeated washing of the disk with the above washing solution. The bound complexes were detected with *p*-nitrophenyl-phosphate (PNP), and the OD of the supernatant was measured with a microtiter plate reader (Titertek, Flowlab) at 405 nm. The results (OD) were compared with four individual sera and with a serum pool of 10 occupationally unexposed subjects.

Competitive immunoabsorption tests

To determine immunologic cross-reactivity of the anhydride-HSA conjugates, we performed immunoabsorption tests. A volume of 10 µl (10 µg) of the anhydride-HSA conjugates PMDA, TMA, and MA was added to the serum of S.M. (40 µl) and tested with the different anhydride-HSA-coated disks in the EAST, as described above.

SDS–PAGE and immunoblotting

The SDS–PAGE was performed according to the manufacturer's instructions with normal human serum pool, anhydride-treated normal serum pool, and the serum of the patient. An 8–25% gel gradient was used in the Phastcast electrophoresis system (Pharmacia, Germany). The precast gels were loaded with 4 µl of either patient's serum, normal serum pool treated with PMDA, or serum pool of 10 unexposed subjects. After dilution to 1:32 in PBS, the Phastcast system was programmed to run for 30 min

at 200 V, 10 mA. After electrophoresis, the proteins were transferred by Western blotting to nitrocellulose (NC) papers, and the immunoblots were developed with the following antisera: 1) antigen: normal serum pool, anhydride-treated serum pool, patient serum/antibody: goat-antihuman serum conjugated to alkaline phosphatase; 2) antigen: same as in 1)/ antibody: murine monoclonal antihuman IgG (Fc) conjugated to alkaline phosphatase; 3) antigen: normal human serum pool, anhydride-treated serum pool/antibody: patient serum or normal serum, followed by murine monoclonal antihuman IgG (Fc) conjugated to alkaline phosphatase; 4) antigen: normal serum pool, anhydride-treated serum pool/ antibody: patient serum/murine monoclonal antihuman IgG1–4/antibody: goat antimouse IgG biotin labeled/streptavidin conjugated to alkaline phosphatase.

Incubation times were 120 min in a shaker water bath at 37 °C. Between the incubations, the NC papers were washed six times with 0.05 M TRIS-HCl, pH 7.5, containing 0.5% Tween-20. In all cases, the blots were visualized with *p*-nitrophenyl-phosphate as substrate.

Results

The EAST of the patient's serum revealed high antibody concentrations of specific IgG against PMDA-HSA and TMA-HSA conjugates. The highest antibody concentration was seen when PMDA-HSA conjugate was used (Fig. 1). The EAST for specific IgG of PA- and MA-HSA was within the normal range; IgE antibodies against these two conjugates could not be detected by EAST.

The immunoabsorption tests revealed a considerable cross-reaction between PMDA-HSA and TMA-HSA antibodies. When soluble PMDA-HSA was added to the serum, a significant reduction of specific IgG antibodies occurred (> 50% inhibition) with TMA-HSA disks. Similar results were obtained with soluble TMA-HSA and PMDA-HSA disks. MA-HSA did not block antibody binding to TMA-HSA disks.

SDS–PAGE and immunoblotting showed differences between serum proteins of unexposed subjects and the serum of patient S.M. with PMDA exposure. Comparison of the serum protein fractions of patient S.M. with the serum proteins of unexposed subjects showed that the serum albumin band of the patient was reduced in intensity, as compared with the normal sera. In this respect, the serum albumin fraction was similar to the normal serum pool after *in vitro* treatment with PMDA. Differences in the IgG fractions were also observed (Fig. 2). With Fc-specific monoclonal mouse antihuman IgG antibodies for the development of the immunoblots, it was shown that *in vitro* treatment of the normal serum pool by PMDA results in high-mol.-mass, cross-linked IgG oligomeres (> 300 kDa) with apparently intact antigen determinants on the Fc portion (Fig. 2, lane 1). The serum of patient S.M. with occupational exposure to PMDA showed two distinct populations of IgG molecules, as compared with the IgG molecules of unexposed subjects (Fig. 2, lines 2 and 3): one IgG species of normal mol. mass (about 150 kDa) and another oligomere at about 250–300 kDa. Thus, the IgG molecules in the serum of S.M. could be interpreted as an intermediate state between untreated IgG and IgG treated with PMDA *in vitro*.

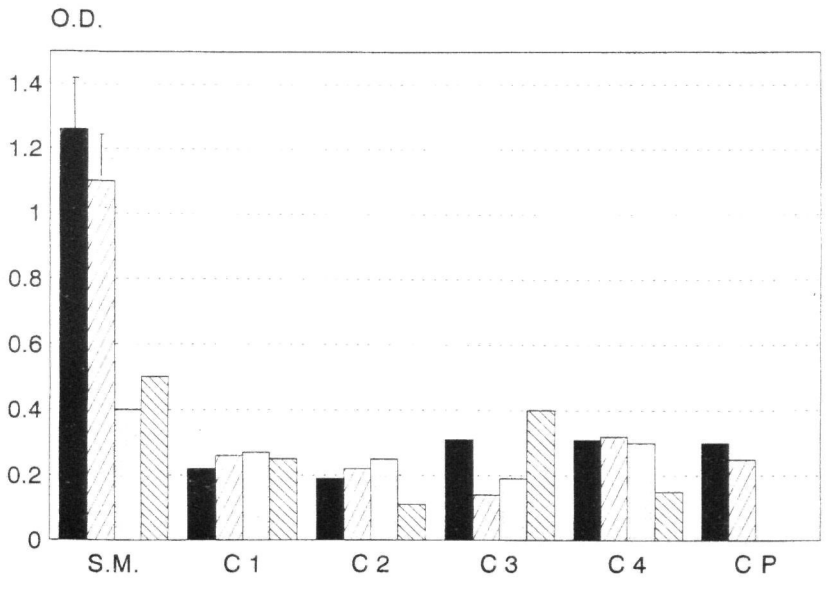

Fig. 1. Comparison of IgG binding of patient S.M. to sera of four unexposed controls (C1–C4) and to serum pool of 10 unexposed controls (CP) in EAST. Following acid anhydride HSA conjugates were used as solid-phase antigens in EAST: PMDA-HSA (■), TMA-HSA (▨), PA-HSA (⁝⁝), and MA-HSA (▧). Binding of IgG to PMDA-HSA in serum of S.M. was significantly higher than controls.

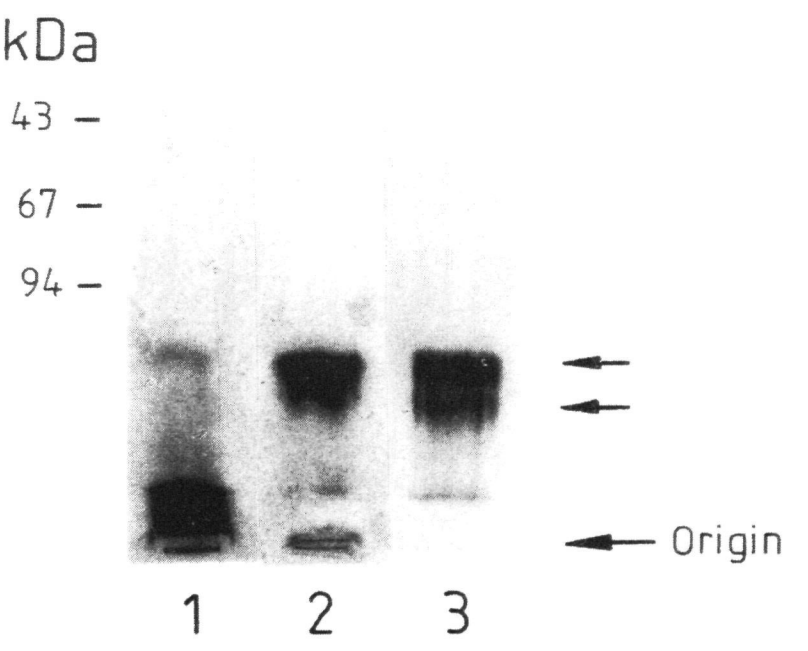

Fig. 2. Immunoblotting of PMDA-treated normal serum pool (1), untreated normal serum pool (2), and serum of patient S.M. (3). Blot was developed with murine monoclonal antihuman IgG (Fc specific). Note two distinct populations of IgG molecules in serum of S.M. at 150–250 kDa (arrows).

In vitro treated human serum with the anhydrides was also immunoblotted with the patient's own serum as the first antibody, and the blots were developed with murine antihuman IgG (Fc). The results show that the patient had a whole spectrum of specific IgG against PMDA-modified serum proteins, other than HSA, in the higher mol. mass range (> 67 kDa), disregarding the IgG fractions in both anhydride-treated serum and untreated serum. The latter are apparently directly recognized by the murine monoclonal anti-IgG (Fig. 3, lanes 1 and 3).

With the immunoblots of PMDA-treated serum and the patient's serum as first antibody, with monoclonal mouse antihuman IgG1, 2, 3, and 4 as second antibody, only the antihuman IgG4 antibody resulted in a positive reaction, indicating the presence of anti-IgG autoantibodies of the subclass IgG4 in the serum of S.M. In our hands, the unconjugated (free) acid anhydrides did not show any reactions in the immunoassays.

The patient recovered completely after leaving his job without any further treatment. He remained clinically asymptomatic for 12 months, and his EAST values returned to normal after 14 months.

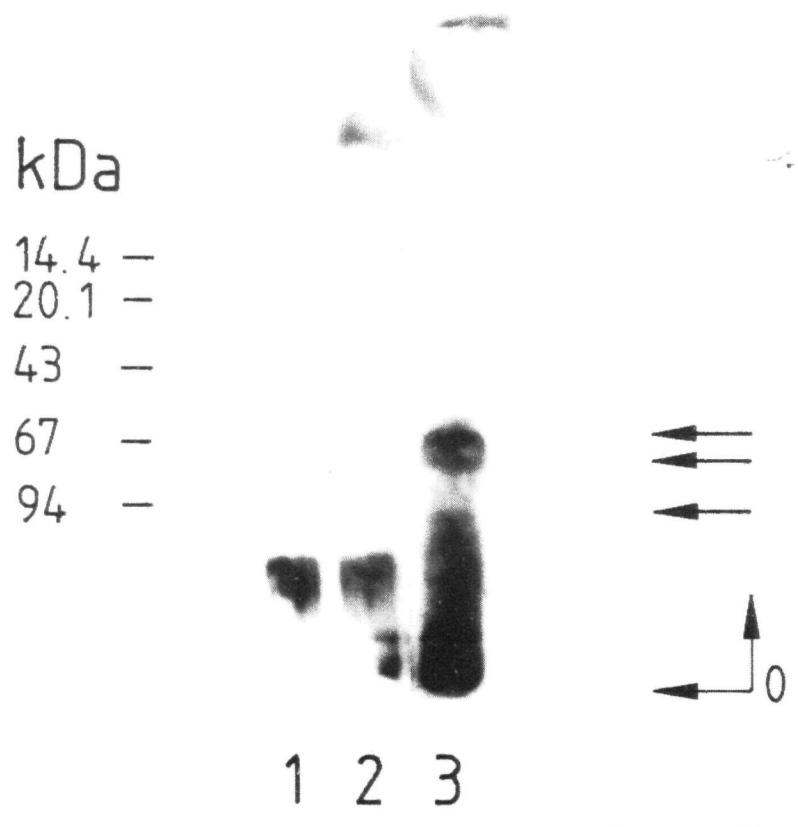

Fig. 3. Immunoblotting of normal serum (1) and (2) and PMDA-treated serum (3). First antibody was normal serum (1), serum of S.M. (2), and serum of S.M. (3). Second antibody: murine monoclonal antihuman IgG. Note various PMDA-treated serum proteins in > 67-kDa range recognized by IgG antibodies in serum of S.M. Similar bands in range > 100 kDa (1) and (2) are due to direct reaction of murine anti-IgG with IgG molecules in samples (1) and (2). PMDA-treated serum (3) with normal serum as first antibody did not show any bands.

Discussion

Hypersensitivity reactions to acid anhydrides are documented in the literature (4, 6, 7). These cases involved bronchial asthma and showed positive skin test results and specific IgE antibodies in sera of persons exposed to various anhydride-HSA conjugates. An exclusively IgG-mediated immunoresponse has so far been demonstrated only in animal experiments (1) and in exposure to TMA (2). This is the first time that workplace exposure to the anhydride PMDA inducing autoantibodies and hemorrhagic alveolitis has been observed.

The immunoabsorption tests with serum of S.M. indicate that a considerable similarity must exist between PMDA- and TMA-modified HSA molecules. Whether this is due to structural similarity of PMDA and TMA (both of which contain an aromatic benzene ring) or to the mode of reactions with HSA cannot be determined at present. The EAST results (Fig. 1) show also that antibody binding to both conjugates is nearly identical. Since S.M. was never exposed to TMA at his workplace, it must be concluded that the cause of his illness was PMDA.

Since he had worked there for a longer period of time, it can be assumed that his symptoms were caused by acute overexposure to PMDA. Because his symptoms disappeared after he left his job and his antibody levels returned to normal, a direct causal relation between PMDA exposure and his illness can be postulated.

In addition to antibodies against a number of PMDA-treated serum protein fractions, the serum of S.M. clearly indicated two different types of IgG molecular species with regard to the Fc regions. It is conceivable that different modified Fc portions of IgG molecules induce complement binding and a complement cascade under unphysiologic conditions, resulting in cell membrane and/or lung tissue damage. This hypothesis is further supported by the elevated autoantibody titers against DNA and cardiolipin, suggesting a secondary autoimmune process. However, since this is the first report of PMDA-induced IgG-mediated hemorrhagic alveolitis, no definite conclusions can be drawn as to the prevalence and incidence of such symptoms.

The lack of results with the free, unconjugated anhydrides is not surprising because acid anhydrides hydrolyze quickly in an aqueous medium, resulting in the corresponding di-, tri-, or tetracarboxylic acids, which are chemically inert and unable to react with any serum proteins. However, this conflicts with the results reported by Topping et al. (5), in which hapten specificity was demonstrated. Whether in the latter case the apparent immunoinhibition was due only to the lowered pH, caused by anhydride-derived di-, tri-, or tetracarboxylic acids after hydrolysis of

the anhydrides, or was indeed due to specific antibodies reacting with the carboxylic acids, has not been investigated. The latter possibility, however, seems to be very unlikely. Individual specificities of antibodies are also possible.

Regarding the results of the immunoblot with anti-human IgG4, the most likely interpretation seems to be that the (auto)antibodies of the patient (IgG4) reacted primarily with *in vitro*-modified serum proteins (i.e., Ig), and the subclass IgG4 of the patient was recognized by murine anti-IgG4, since no reaction could be detected with normal serum IgG4 as antigen. The results of the anti-DNA and anti-cardiolipin antibodies would also indicate an autoimmune process.

Increased levels of specific IgG have been found against various isocyanates, resulting in hypersensitivity pneumonitis. Whether the findings described here are specific for hemorrhagic alveolitis cannot be decided at this stage because this is the first report of such a case. However, in a large study at a German chemical plant (Czuppon et al., unpublished), no specific IgG with these clinical data has been found despite about 13% hypersensitivity (IgE) in the workers. This would also support the hypothesis that IgE-mediated hypersensitivity to acid anhydrides results in a specific clinical manifestation.

Further investigations are now underway to identify the various serum protein fractions, other than HSA and IgG, that are modified by PMDA after *in vitro* treatment.

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