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Participation of eosinophils in the toxic oil syndrome

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SUMMARY

The participation of eosinophils in the Spanish toxic oil syndrome (TOS) was investigated. Eosinophil infiltration and degranulation in tissues from 52 patients with the TOS were examined by immunofluorescence staining for the eosinophil granule major basic protein (MBP). Serum MBP levels were determined in sera from 323 patients. Eosinophil infiltration and degranulation were found in several tissues, especially during the acute phase of the TOS, and serum MBP was significantly elevated during all phases of the disease, suggesting that eosinophils play a role in the pathogenesis of the TOS.

Keywords eosinophils toxic oil syndrome eosinophil granule major basic protein

INTRODUCTION

In May 1981, a previously unknown disease occurred in Spain, which was finally attributed to the ingestion of adulterated rapeseed oil (Grandjean & Tarkowski, 1984a). Patients with this disease, termed the toxic oil syndrome (TOS), presented with acute respiratory symptoms, followed by an intermediate or subacute phase 1–3 months later, characterized by intense myalgias, thromboembolism (Castro Garcia *et al.*, 1986), weight loss, and sicca syndrome, and lastly a chronic phase, commencing at approximately 4 months, characterized by scleroderma-like lesions, peripheral neuropathy, muscular atrophy and pulmonary hypertension (Toxic Epidemic Syndrome Study Group, 1982; Kilbourne *et al.*, 1983).

Despite the well-proven relation between one of the oil compounds (fatty acid anilides) and the risk of illness (Kilbourne *et al.*, 1988), an animal model has not been developed (Grandjean & Tarkowski, 1984b). Other products proposed, such as 1-phenyl-5-vinyl imidazolidine-2-thione and vinyl chloride (Kammuller, Penninks & Seinen, 1984; Hernandez Bronchud, 1984), have not been detected in oils related to the clinical cases (Hernandez Bolanos, 1982; Bernert *et al.*, 1989), and therefore can not be directly implicated in the development of symptoms. The pathogenesis of the disease is still unclear, and different mechanisms of tissue damage have been proposed, including contributions of leukotrienes (Garcia Gil *et al.*, 1984), free radicals and immunologic reactions (Vicario *et al.*, 1982; Gutierrez *et al.*, 1983; Lahoz *et al.*, 1983).

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Vascular endothelium seems to be the target tissue in the TOS (Martinez-Tello *et al.*, 1982; Ricoy *et al.*, 1983). One hypothesis assumes direct toxic effects on tissues followed by immunologic reactions perpetuating the injury and leading to fibrosis in different organs (Posada *et al.*, 1987). A characteristic feature of the TOS in at least 80% of the patients was peripheral blood eosinophilia, with levels higher than 1500 cells/mm^3. On this basis, eosinophils are suspects in the pathogenesis of the TOS. Presently, relatively little is known about the involvement of eosinophils in tissue damage during different stages of the TOS. Eosinophilia was present during the acute phase of the TOS and eosinophil granules were present in muscle biopsies (Ricoy *et al.*, 1983). In another study, however, eosinophil maturation appeared normal, but the cells themselves showed only multiple abnormal lipid vacuoles (Gabriel *et al.*, 1986). In order to elucidate the potential role of eosinophils in the TOS, we tested whether eosinophil degranulation occurred in tissues by immunofluorescence localization of the eosinophil granule major basic protein (MBP) and by measurement of the levels of MBP in the sera of TOS patients. We found eosinophil infiltration, as well as eosinophil degranulation in several tissues, predominantly during the acute phase of the disease. The serum levels of MBP were above normal in 23–45% of patients, depending on the stage of the disease. These results suggest that eosinophils may infiltrate certain tissues during early stages and degranulate there, releasing toxic proteins into tissues.

MATERIALS AND METHODS

Sera

A total of 323 sera from affected patients at two different hospitals in Madrid, as well as 103 sera from normal blood

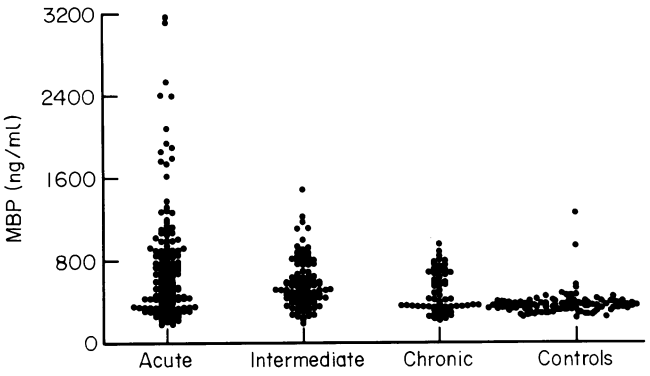

Fig. 1. Serum levels of major basic protein (MBP) as measured by radioimmunoassay in 103 healthy blood donors and in 323 patients from the three phases of the toxic oil syndrome (TOS), acute, subacute or intermediate, and chronic. Mean MBP levels (ng/ml) ± s.e.m. and numbers of sera examined for each of the four patient groups were as follows: acute, 720 ± 42, $n = 159$; subacute, 579 ± 24, $n = 100$; chronic, 482 ± 24, $n = 64$; and healthy, 368 ± 12, $n = 103$. Only 5% of healthy persons have values higher than 608 ng/ml.

donors, were tested. Samples were stored at −20°C from 1981 until the time of the study. One-hundred and fifty-nine sera were from patients in the acute phase, defined as the presence of interstitial–alveolar pulmonary infiltrates and/or pleural effusion on chest roentgenograms during the 2 months after the onset of the symptoms. One-hundred sera were from patients in the subacute phase of the TOS who were subjected to a therapeutic program with vitamin E (anti-free radicals), but never treated with glucocorticoids. Finally, 64 sera were obtained from patients in the chronic phase of the TOS. Patients were of both sexes and all ages. Normal plasma was collected from 52 consecutive male and 51 consecutive female healthy blood bank donors at the Mayo Clinic.

Radioimmunoassay for MBP

MBP levels were measured in the serum by a double-antibody radioimmunoassay (Wassom *et al.*, 1981). Sera were frozen at −20°C and shipped from Spain to Rochester, MN, on dry ice. MBP levels were measured after dilution of the samples in Tris–EDTA buffer (0·15 M NaCl, 0·01 M EDTA, 0·33 M Tris, pH 8) and reduction and alkylation in 7·5 mM dithiothreitol and 15 mM iodoacetamide, respectively. Samples were diluted in PPF-E buffer (0·1 M Na_2HPO_4, 0·1% protamine, 0·01 M EDTA, 0·5% fetal calf serum, 0·1% NaN_3, pH 7·5) and incubated with 100 μl of rabbit anti-human MBP (1/5000 dilution in PPF-E) for at least 20 min at 22°C. MBP, 0·5 ng, labelled with ^{125}I by the chloramine-T method (Greenwood, Hunter & Glover, 1963), was added to each sample and incubated overnight at 4°C. The MBP–anti-MBP complexes were precipitated with 100 μl of burro anti-rabbit IgG and 100 μl of normal rabbit serum diluted 20-fold in PPF-E, followed by centrifugation at 850 ***g*** for 15 min. The supernatants were decanted, and the sediments counted in a gamma scintillation counter. Every sample was analysed in duplicate, and the amount of MBP per tube was calculated by comparing the ct/min obtained to a dose–response curve of reduced and alkylated purified MBP. The coefficient of variation of a normal serum included in each assay was 35·2%; for a high MBP serum the coefficient of variation was 15·6%.

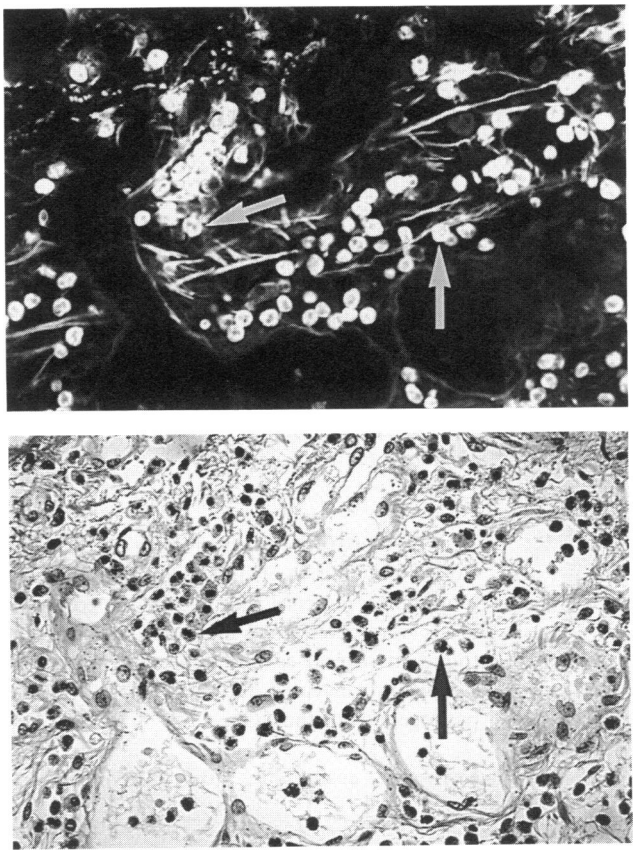

Fig. 2. Immunofluorescence localization of major basic protein (MBP) in lung tissue from a patient in the acute phase of the toxic oil syndrome (TOS). Top, section stained with rabbit anti-human MBP; bottom, haematoxylin/eosin counterstain of the section above. Note the presence of intact eosinophils infiltrating the tissue (arrows). Extracellular MBP is also localized in linear structures. Section stained with normal rabbit IgG showed no significant staining. Magnification ×260.

Immunofluorescence staining for MBP

Fifty-six tissue specimens obtained at autopsy from 52 patients were stained for MBP by a method previously described (Filley *et al.*, 1982; Peters *et al.*, 1983). Briefly, the tissues were fixed in 10% formalin, and embedded in paraffin for storage. Sections of 6 μm were mounted on slides coated with LePage's glue, deparaffinized in xylene, rehydrated, digested with 0·1% trypsin solution, pH 7·8, for 1 h at 37°C, washed in water and incubated in 10% heat-inactivated normal goat serum in phosphate-buffered saline (PBS) at 4°C overnight. After a wash in PBS, slides were overlaid with either protein A-purified normal rabbit IgG as a negative control, or affinity chromatography-purified rabbit anti-human MBP polyclonal antibody, in a humidified chamber at 37°C for 30 min. After another wash with PBS, slides were incubated with a 1% solution of chromotrope 2R (EM Science, Cherry Hill, NJ) for 30 min at 22°C and washed again. They were then overlaid with fluorescein-conjugated goat anti-rabbit IgG (Cappel Research Reagents, Malvern, PA) in a humidified chamber at 37°C for 30 min, washed with PBS, mounted with 10% PBS/90% glycerol solution containing *p*-phenylenediamine (Aldrich Chemical Company, Milwaukee, WI) (Krenik *et al.*, 1989), cover-slipped and sealed with clear nail polish. Slides were examined with a Zeiss standard micro-

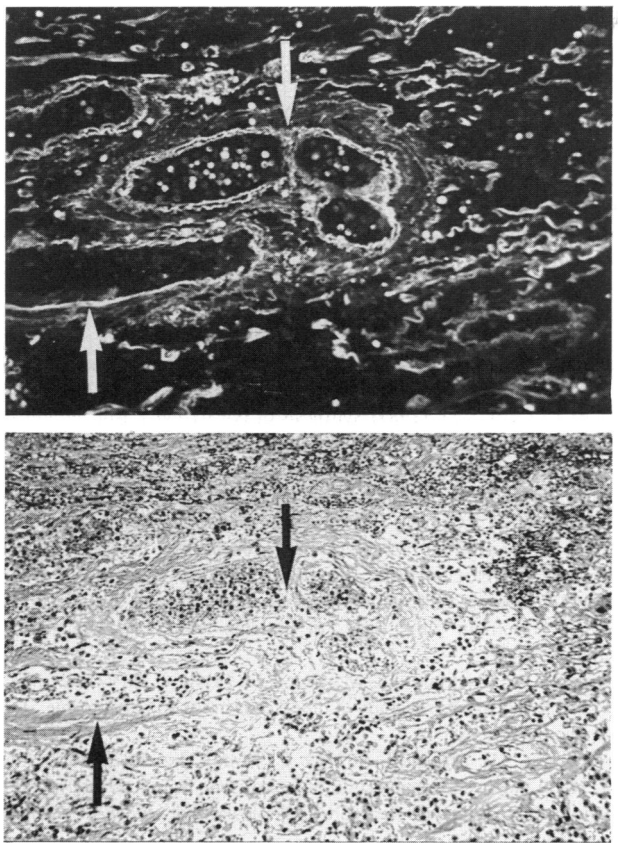

Fig. 3. Immunofluorescence localization of major basic protein (MBP) in lung tissue from a patient in the acute phase of the toxic oil syndrome (TOS). Top, section stained with rabbit anti-human MBP; bottom, haematoxylin/eosin counterstain of the section above. Note the extensive deposition of extracellular MBP in the vessel wall (arrows) and the perivascular areas. A few intact eosinophils are present mainly in the vessel lumen. Section stained with normal rabbit IgG showed no significant staining. Magnification × 100.

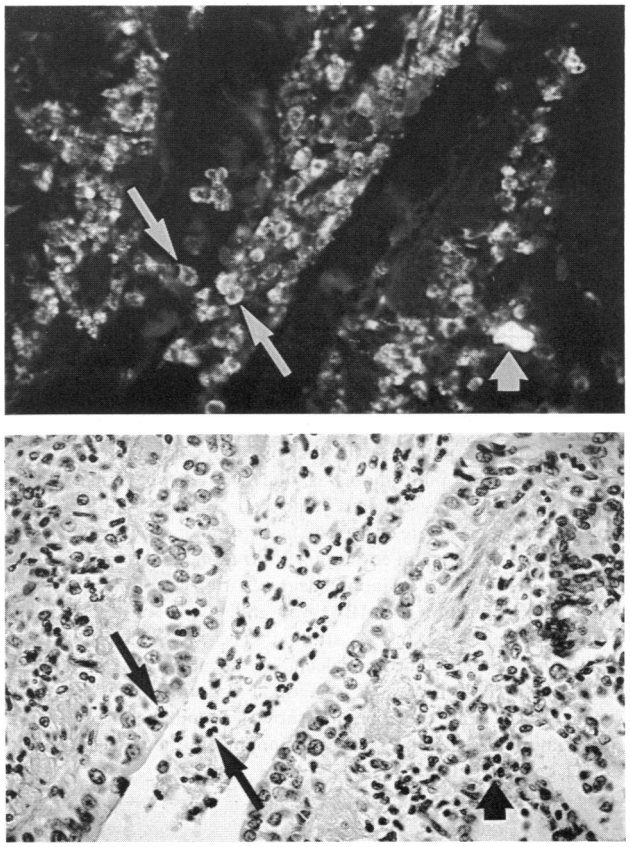

Fig. 4. Immunofluorescence localization of major basic protein (MBP) in lung tissue from a patient in the acute phase of the toxic oil syndrome (TOS). Top, section stained with rabbit anti-human MBP; bottom, haematoxylin/eosin counterstain of the section above. Note the presence of a clump of eosinophils that stains brightly (arrowhead) and numerous neutrophils (arrows) and occasional mononuclear cells that stain weakly. Section stained with normal rabbit IgG showed no significant staining. Magnification × 260.

scope with vertical illumination for epi-fluorescence and a fluorescein filter system (Carl Zeiss, Thornwood, NY). Slides stained with rabbit anti-human MBP were counterstained later with haematoxylin–eosin.

Statistical analysis

The statistical analysis of the serum samples was done by Student's *t*-test on a Hewlett-Packard 9845T computer with general statistics program no. 09845-15030. Comparison of eosinophil infiltration and degranulation between tissues from patients with acute and chronic TOS was performed by the rank sum test.

RESULTS

Serum levels of MBP

The serum levels of MBP were compared in the acute, subacute and chronic phase to normal controls (Fig. 1). In the acute phase 45% of the samples were more than two standard deviations above the mean of normal sera. By contrast, 32% and 23% of the samples from the subacute and chronic phases, respectively were elevated. Mean serum levels of MBP in each of the three phases of the TOS were significantly elevated ($P<0{\cdot}001$) compared with the controls.

Immunofluorescence localization of MBP

In tissues from certain patients (Fig. 2), MBP was localized mainly in intact eosinophils, whereas in others the predominant MBP staining was due to extracellular deposition (Fig. 3). Six of 27 lung specimens showed a linear deposition of MBP similar to that seen in Figs 2 and 3. In some cases, very brightly stained cells co-existed with weakly stained cells that were eosinophils and neutrophils, respectively, by haematoxylin–eosin counterstain (Fig. 4). Most of the tissues showed evidence of eosinophil infiltration and/or eosinophil degranulation, regardless of the phase of the disease. However, comparison of tissues from patients with the acute and chronic phases indicated that samples from the acute phase generally showed more striking eosinophil participation ($P<0{\cdot}03$) (Fig. 5).

DISCUSSION

The presence of peripheral blood eosinophilia was a common finding in patients with TOS. To study the participation of the

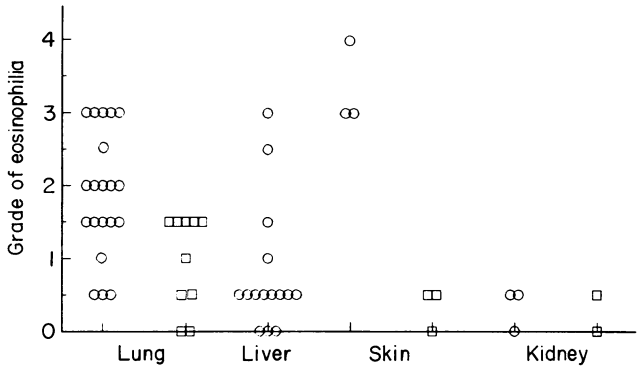

Fig. 5. Summary of mean grades of eosinophil infiltration and/or extracellular deposition of major basic protein (MBP) in 56 tissue specimens from 52 patients with the toxic oil syndrome (TOS). The degree of eosinophil infiltration was graded 1–4:1, occasional eosinophils; 2, few eosinophils scattered in the tissue, occupying < 10% of the tissue section; 3, moderate eosinophil infiltration occupying 10–25% of the tissue; and 4, abundant eosinophil infiltration, occupying > 25% of the tissue. The degree of eosinophil degranulation (free granules and/or extracellular deposition of MBP) was graded 1–4:1, occasional; 2, moderate; 3, marked; and 4, striking. The final grade for tissue eosinophilia was determined by calculating the mean between the grade of eosinophil infiltration and eosinophil degranulation. Note that normal tissues from these organs show no evidence of eosinophil degranulation and only occasional eosinophils in the tissues. O, acute TOS; □, chronic patients.

eosinophil in tissue damage during the different stages of TOS, several tissues were stained by immunofluorescence, using a specific antibody for eosinophil granule MBP. Intact eosinophils and eosinophil degranulation were present in tissues during every stage of the disease, but most strikingly during the acute phase. In addition, many patients had increased serum MBP. MBP levels are quite stable in stored serum; nonetheless, prolonged storage causes slight drops in serum levels (Wassom *et al.*, 1981). These results suggest that eosinophils participate in the pathogenesis of TOS because eosinophil granule proteins are toxic to parasites and mammalian cells (Gleich *et al.*, 1979; Gleich & Adolphson, 1986). The different patterns of immunofluorescence staining suggest the existence of a cascade of events: (i) intact eosinophils infiltrate the tissues attracted by a chemotactic factor released during the inflammatory reaction or by some component or metabolite of the toxic oil; (ii) eosinophils are activated, possibly by a cytokine such as tumour necrosis factor (Silberstein & David, 1986) and degranulate, releasing cationic proteins such as MBP, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin, all of which are toxic to mammalian cells (Gleich & Adolphson, 1986; Nakajima, Loegering & Gleich, 1988); (iii) extracellular deposits of eosinophil granule proteins are phagocytosed by other cells, thus accounting for the weak staining of neutrophils and mononuclear cells in areas where eosinophils are also present; (iv) eosinophil granule proteins are not only toxic to mammalian cells by themselves, but can also play a role in hypersensitivity reactions, by inducing mast cell degranulation (Zheutlin *et al.*, 1984) or by modulating the effect of leukotrienes (Goetzl, 1982); and (v) the elevation of serum MBP might be due to degranulation of some eosinophils in the bloodstream, before entering the tissues, or to reabsorption of MBP deposits from the tissues to the bloodstream.

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