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Case report

Immunopathological study of eosinophils in eosinophilic granuloma of bone: evidence for release of three cationic proteins and subsequent uptake in macrophages

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Summary. Eosinophils from two patients with eosinophilic granuloma of bone (EGB) were studied by combined immunohistochemical and immuno-ultrastructural methods with antibodies directed against three eosinophil granule proteins: major basic protein, eosinophil cationic protein, and eosinophil peroxidase. Immunohistostaining showed the presence and distribution of large numbers of eosinophils in the granuloma. Immuno-ultrastructural methods showed alterations of eosinophil fine structure associated with some steps in the release of granule proteins. No granule extrusion was seen, but rather cationic proteins diffused within cytoplasmic tubulo-vesicular structures. Furthermore, the three granule proteins were found within phagolysosomes of surrounding macrophages, suggesting an interaction between eosinophils and phagocytic cells at the destructive stage of EGB.

Key words: Eosinophilic granuloma of bone – Eosinophil granule proteins – Immunostaining – Ultrastructure

Introduction

Eosinophilic granuloma of bone (EGB) was first described in 1940 by Lichtenstein and Jaffé (1940). It belongs to the group of Langerhans cell histiocytoses (Chu et al. 1987) and is considered a localized, benign form. The granuloma, composed of histiocytes (most of them Langerhans cells, as demonstrated by Nezelof et al. in 1973), numerous eosinophils, and lymphocytes, is associated with bone destruction (Oberman 1961; Favara et al. 1983). Three successive pathological stages can be observed: an initial red lesion with histiocytes and numerous eosinophils, followed by a yellow lesion mostly composed of lipid-laden macrophages but no more eosinophils, and a final grey cicatricial lesion with fibroblasts and dense collagen bundles (Engelbreth-Holm et al. 1944).

Only one recent paper by Zabucchi et al. (1991) has focused on eosinophils in EGB, demonstrating extensive degranulation and release of eosinophil peroxidase (EPO). The role of eosinophils in IgE-dependent hypersensitivity mechanisms (Capron et al. 1984, 1989), cytotoxic reactions (Gleich et al. 1979; Gleich and Adolphson 1986; Samoszuk et al. 1988; Motojima et al. 1989) and in collagen remodelling (Basset 1983; Pincus et al. 1987) is now well documented. The main mediators of these effects, now characterized (Spry 1988), are cationic proteins present in eosinophil granules, eosinophil major basic protein (MBP), eosinophil cationic protein (ECP), EPO and eosinophil-derived neurotoxin, now well characterized. In vitro assays have suggested that these cationic proteins could be selectively released, after stimulation of various Fc receptors (Khalife et al. 1986; Tomassini et al. 1991) or triggering of membrane components (Tai et al. 1986). However, the precise mechanism of release of granule components has not yet been elucidated. The Charcot-Leyden crystal protein (CLC), biochemically distinct from MBP (Ackerman et al. 1980), is the only constituent of Charcot-Leyden crystals. Since it is present in intact eosinophils (Weller et al. 1981), but outside eosinophil granules (Dvorak et al. 1991), detection of CLC is a good marker of eosinophil lysis.

In the present paper, we studied eosinophils during the initial, destructive stage of two cases of EGB. We used antibodies directed against three eosinophil granule proteins (MBP, ECP, EPO), to detect these effector proteins within the granuloma, and compared their distribution and uptake by macrophage with the distribution and uptake of CLC. A method combining ultrastructure and immunogold-labelled antibodies allowed us to study in vivo the fine structural changes of the eosinophil, associated with release of the granule proteins. We also found strong staining of these proteins within macrophagic compartments, which may suggest an interaction between eosinophils and phagocytic cells at the destructive stage of EGB.

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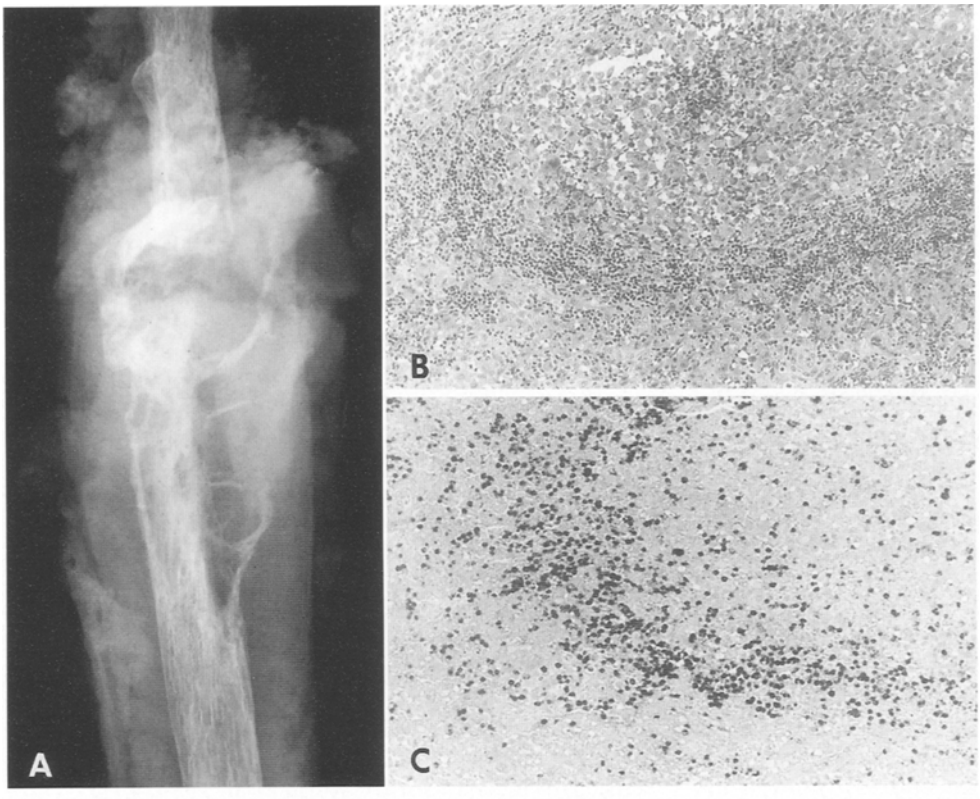

Fig. 1. **A** Radiograph of the damaged rib: multiple well-demarcated osteolytic lesions. **B** Histiocytic proliferation surrounded by a lymphocytic infiltrate mixed with eosinophils. ×250. **C** Intense eosinophilic infiltration revealed by anti-eosinophil cationic protein (ECP) antibody, in an area similar to **B**. Indirect alkaline phosphatase, ×300

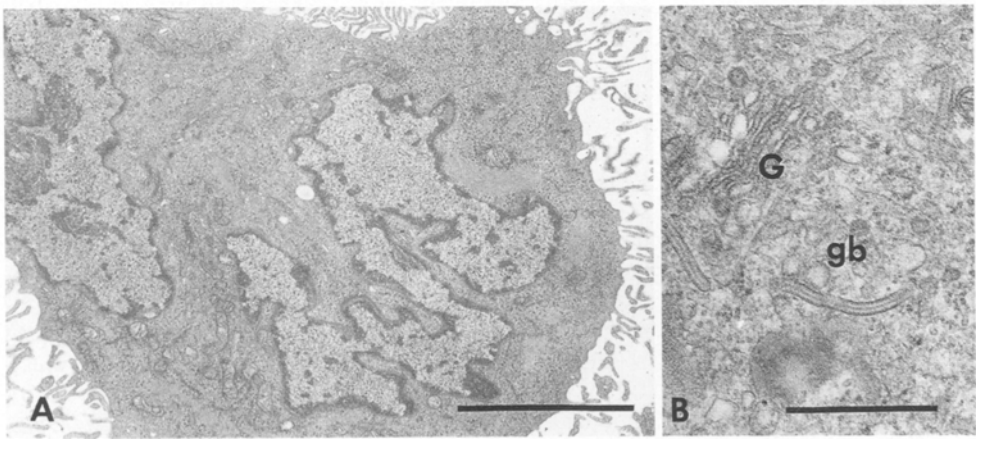

Fig. 2. **A** Ultrastructural aspect of a Langerhans cell; *bar* = 5 μm. **B** High magnification of a typical Birbeck granule (*gb*) observed in the Langerhans cell. *G*, Golgi; *bar* = 0.5 μm

Case reports

Case 1

A 26-year-old man was admitted with a large, painless swelling localized to the right fifth, rib without local skin involvement. Radiological examination showed a well-demarcated lytic defect (Fig. 1A). One year previously the patient had had a pneumothorax resulting from the rupture of an apical emphysematous bulla. A chest radiograph showed a pulmonary infiltrate with a diffuse, mottled pattern. Blood examinations were normal, without eosinophilia. After surgical resection of the damaged rib, pathological examination showed a 10-cm-wide lytic lesion with friable red material containing numerous eosinophils, lymphocytes and histiocytes (Fig. 1A) expressing CD1, CD4, S100 protein and vimentin. Electron microscopic examination showed Langerhans cells (Fig. 2A), with Birbeck granules (Fig. 2B). There was no recurrence of the tumour during a follow-up of 2 years.

Case 2

An 18-year-old man was admitted with a left orbital tumour that had been present for 4 months. It was not painful and did not involve the skin. Skull radiographs showed a 2-cm orbital lytic defect with sharp borders. Another small lesion was found in the right mandible. There was no peripheral blood eosinophilia. Surgical biopsies obtained red, friable material with the same histological and ultrastructural characteristics as in case 1. The two lytic lesions disappeared with radiotherapy and no recurrence was noted in 3 years of follow-up.

Materials and methods

Immunolabelling was performed on cryostat sections from both tumours. Affinity-purified rabbit antibodies to human EPO (kindly provided by Dr. P. Venge, Uppsala, Sweden), and to CLC (kindly

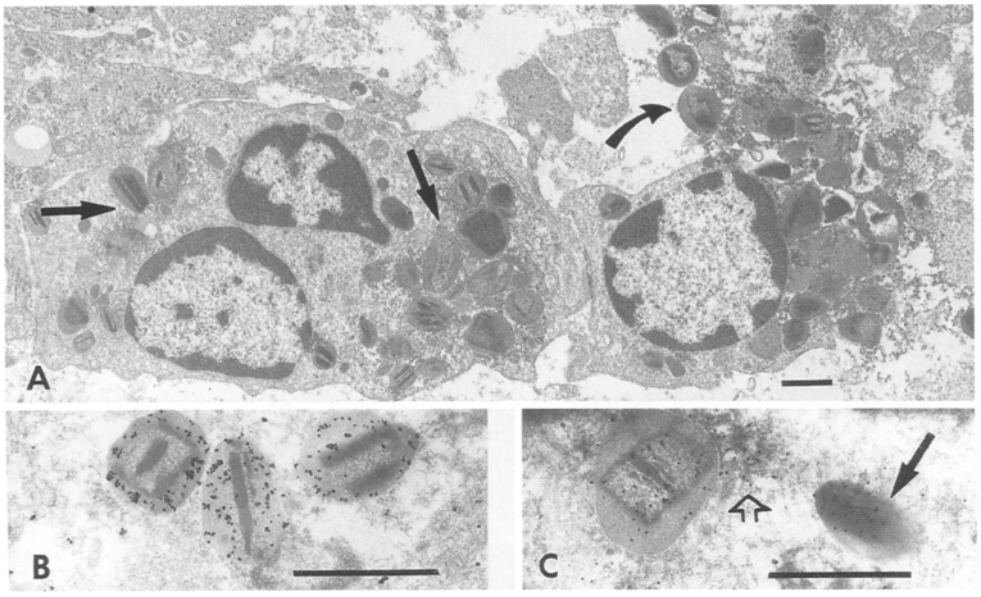

Fig. 3. **A** Ultrastructure of eosinophil changes: alterations of intracytoplasmic granules (*straight arrows*), or cytoplasmic lysis associated with altered granules (*curved arrow*); *bar* = 1 μm. **B** Immunogold labelling with anti-eosinophil peroxidase (EPO) antibody: gold particles are located in the granular matrix; *bar* = 1 μm. **C** Immunogold labelling with anti-major basic protein (MBP) antibody: gold particles are located in the unaltered dense granule core (*arrow*) and in extragranular material surrounding an altered granule (*hollow arrow*); *bar* = 1 μm

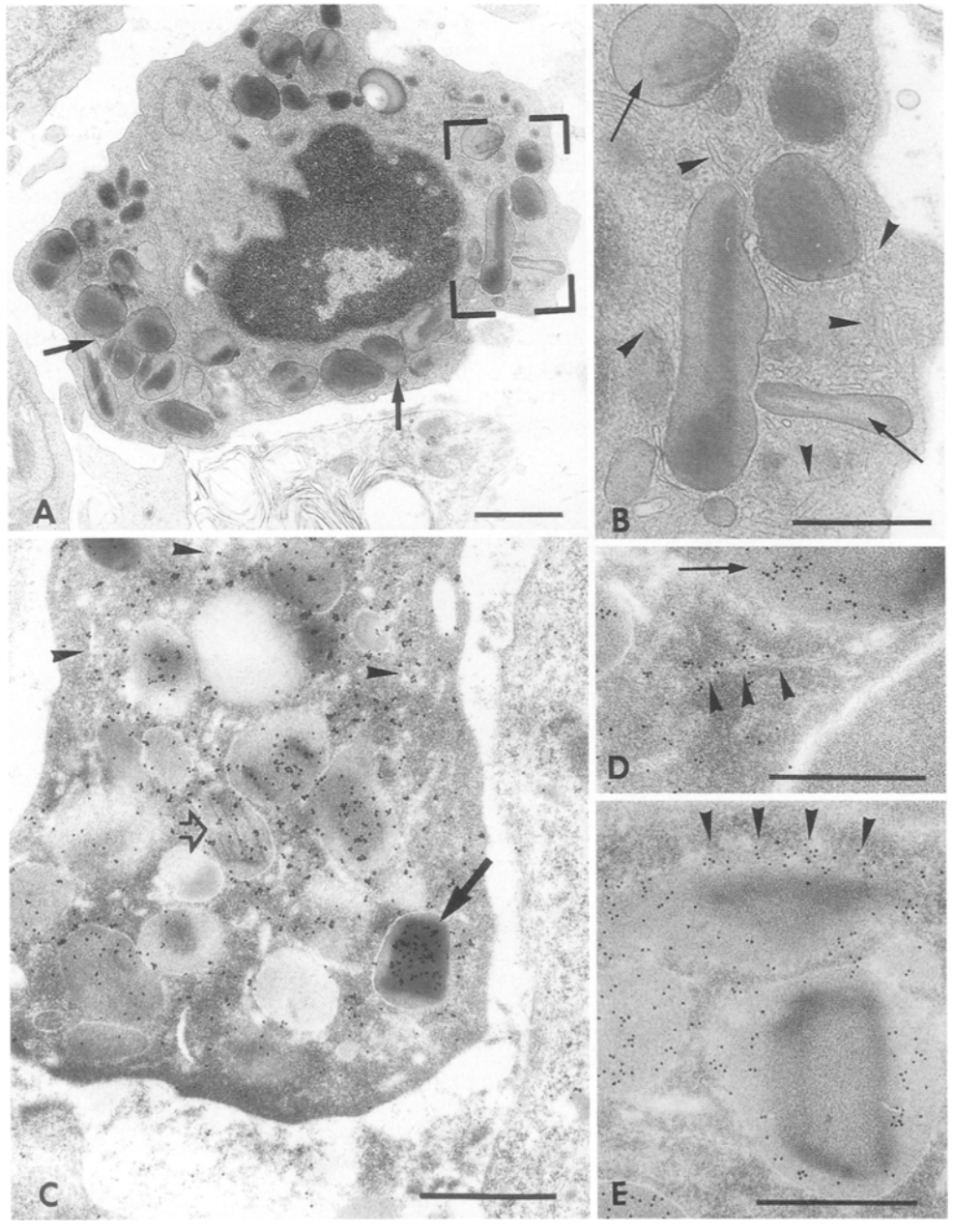

Fig. 4. **A** Ultrastructure of a nonlytic eosinophil: granules are clustered (*arrows*) and some of them have an inverted central core; *bar* = 1 μm. **B** Higher magnification of the framed area of **A**. Besides inverted density of the central cores (*arrows*), numerous tubulo-vesicular structures (*arrowheads*) extend in the cytoplasm; *bar* = 0.5 μm. **C** Immunogold labelling with anti-MBP antibody: dense label of an unaltered central core (*arrow*) and scattered gold particles in an altered central core (*hollow arrow*) and tubulovesicular cytoplasmic structures (*arrowheads*); *bar* = 1 μm. **D** Immunogold labelling with anti-EPO antibody: gold particles are located both in the granular matrix (*arrow*) and in tubulo-vesicular cytoplasmic structures (*arrowheads*); *bar* = 0.5 μm. **E** Immunogold labelling with anti-ECP: gold particles are located within the granular matrix and on the ruffled border of an altered granule (*arrowheads*); *bar* = 0.5 μm

provided by Dr. G.J. Gleich, Rochester, USA), mouse monoclonal antibodies to MBP (Sanbio, The Netherlands), and to ECP (EG_2, kindly provided by Dr. Po Chun Tai, London, UK) were used as primary reagents in an indirect alkaline phosphatase method.

Small specimens of both tumours were fixed in 1% glutaraldehyde in a cacodylate buffer at 4° C. For conventional electron microscopy, aldehyde fixation was followed by osmium tetraoxide fixation, and resin embedding in araldite. For immunocolloidal gold labelling specimens were embedded in Lowicryl K4M according to the standard procedure of Roth et al. (1981).

Ultra-thin sections on nickel grids were incubated for 10 min on a drop of TRIS-HCl buffered saline (20 mM TRIS HCl, 0.5 M NaCl, pH 7.4; TBS) containing 5% (w/v) ovalbumin (OVA), supplemented with 1% heat-inactivated normal goat serum. This was followed by incubation with affinity-purified rabbit antibodies to human EPO, and mouse monoclonal antibodies to MBP or to ECP used as primary reagents. The grids were then rinsed with TBS-OVA and incubated on a drop of the 10 nm gold-conjugated goat anti-rabbit or mouse IgG (1:40) (Janssen Pharmaceutica, Beerse, Belgium). After 1 h incubation at room temperature, sections were thoroughly washed with TBS, post-fixed for 10 min in TBS containing 2% glutaraldehyde and washed again with distilled water. Finally, the sections were subjected to silver enhancement according to a modification of Danscher's silver lactate hydroquinone physical developer (Moeremans 1988). The specificity of the immunostaining was tested by (a) omitting the first antibody, (b) substituting the specific antibodies with the pre-immune serum or with unrelated antibodies whose labelling had been analysed previously (Dissous et al. 1990). Lowicryl sections were stained with uranyl acetate and lead citrate before examination with a Philips EM 420 electron microscope.

Results

The results were similar in the two cases. Immunohistological staining showed that eosinophils were more numerous than expected from May Grunwald Giemsa staining (Fig. 1B), since most of them were mixed with lymphocytes and distributed more around than within histiocytic areas (Fig. 1C). Comparative studies showed no significant difference in the number or in the distribution of cells stained with antibodies against MBP, ECP, EPO, or CLC.

Ultrastructural study showed two types of alterations in eosinophils (Fig. 3A). Approximately 20% of the eosinophils had cytoplasmic lysis associated with altered granules; the other 80% had only alterations of intracytoplasmic granules, some of them with inverted density. Immunogold-labelled EPO and ECP were found in their normal location in the granular matrix, as illustrated for EPO in Fig. 3B. However, few MBP deposits were seen in their normal location in the central cores. Most of the central cores were electron lucent, indicating a loss of MBP deposits. Gold-labelled MBP was mainly located over extragranular material surrounding these altered granules (Fig. 3C).

Eosinophils with no sign of cytoplasmic lysis had their granules clustered in some cytoplasmic areas (Fig. 4A). At higher magnification, their central cores had lost their clear-cut borders and were of irregular density, some of them having an inverted density. Moreover, numerous tubulovesicular structures were present in the cytoplasm between the altered granules (Fig. 4B). Immunogold-labelled MBP was detected in the granule dense core (with higher concentration of gold particles on intact granules than on granules with inverted density), but also in cytoplasmic areas between the granules (Fig. 4C). ECP and EPO were localized in the granule matrix, on ruffled borders of altered granules (Fig. 4E), and in cytoplasmic tubulovesicular structures outside the granules (Fig. 4D).

Interaction of eosinophils with other cell types in the granuloma could also be detected. Eosinophils were

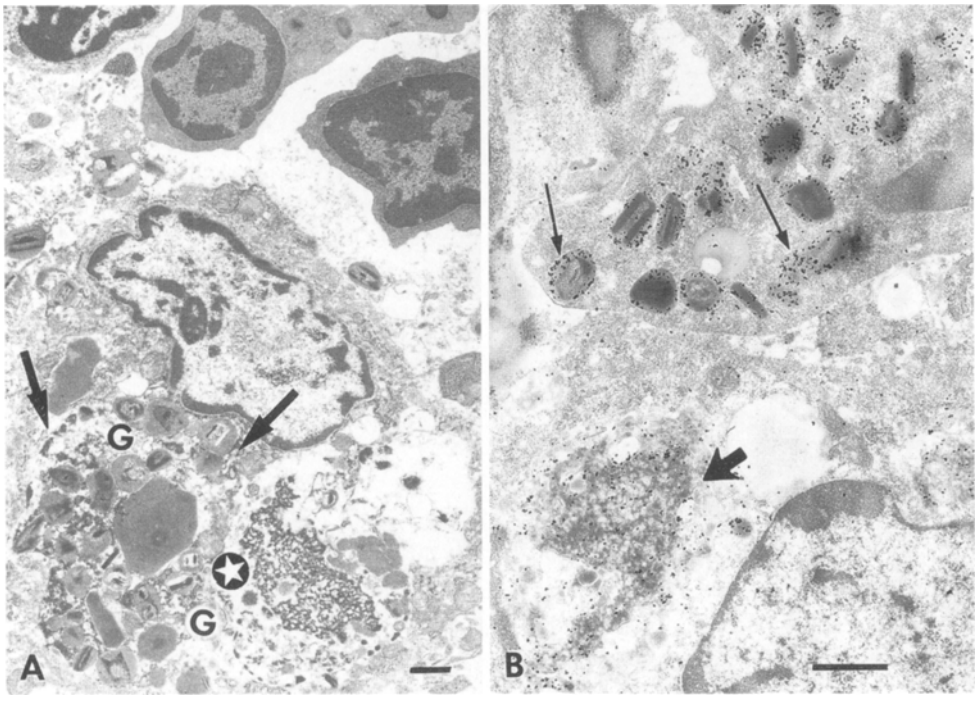

Fig. 5. A Ultrastructure of phagocytosed eosinophil components (*arrows*). Besides altered eosinophil granules (*G*), presence of an electron-dense structure, which appeared to form a network of amorphous material (*star*); *bar* = 1 μm. **B** Immunogold labelling with anti-EPO antibody: EPO is observed in its normal localization in the granular matrix of intact eosinophils (*thin arrows*), but also around degranulated or damaged eosinophils, and in phagolysosome of surrounding macrophages (*thick arrow*). Silver amplification; *bar* = 1 μm

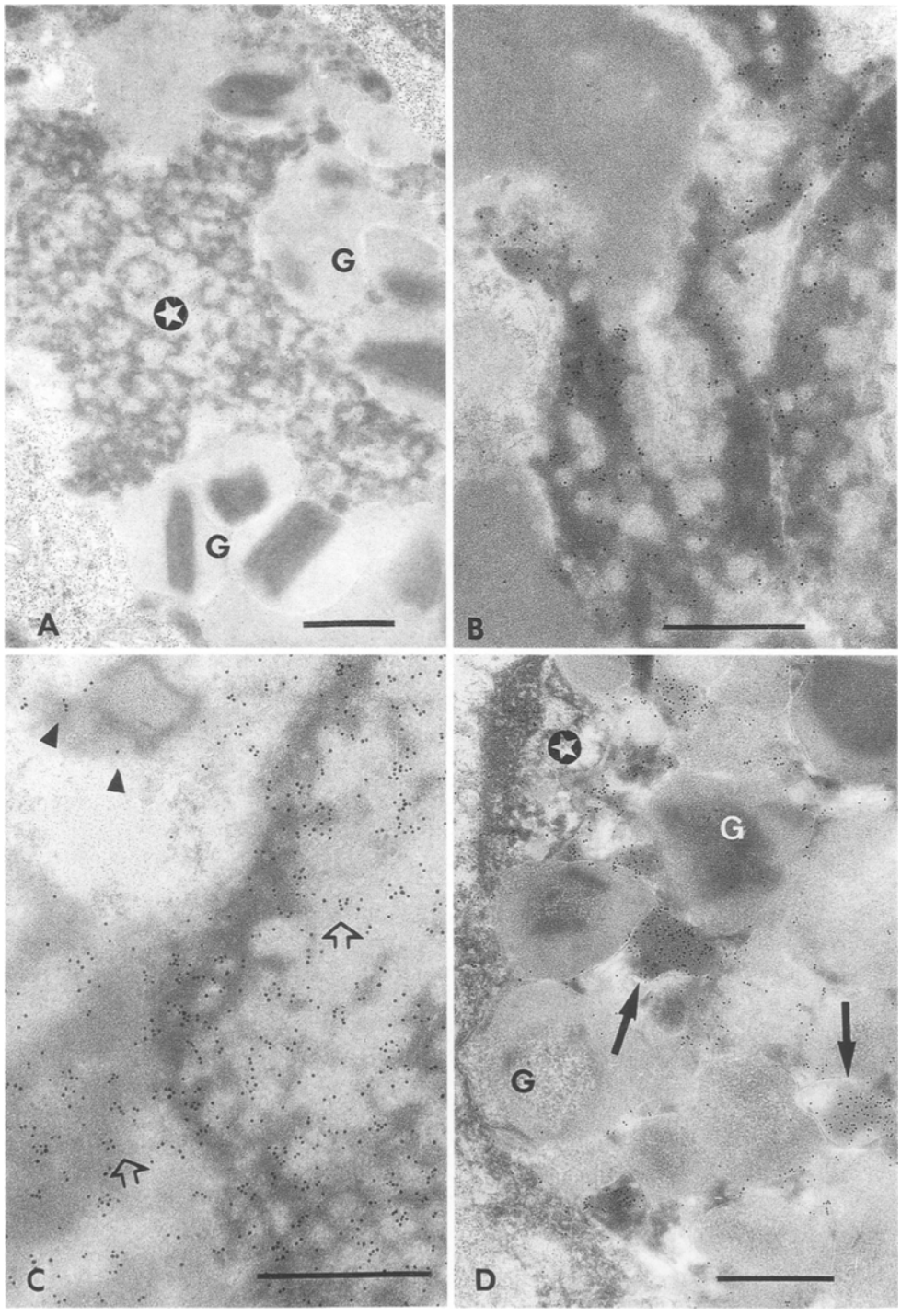

Fig. 6. **A** Higher magnification of the phagocytic vacuoles with altered eosinophilic granules (*G*) aggregated around the electron-dense network (*star*). **B** Immunogold labelling with anti-MBP antibody: gold particules are exclusively associated with this electron-dense network. **C** Immunogold labelling with anti-ECP antibody shows that phagocytosed ECP is found around altered granules (*arrowheads*), on grey areas of the same density (*hollow arrows*), some of them being located within the network. **D** Normally localized within eosinophil cytoplasm, Charcot-Leyden crystal protein, when phagocytosed, is detected on areas (*arrows*) distinct from the eosinophil granules (*G*), and from the electron-dense network (*star*). *Bars* = 0.5 μm

mixed with lymphocytes and numerous cells of the histiocytic lineage: Langerhans cells, macrophages loaded with different kinds of lipids and osteoclasts. No staining of eosinophil granules or ECP in Langerhans cells or lymphocytes was found. However macrophages often contained phagocytosed eosinophil components, as altered eosinophil granules (Fig. 3A). An electron-dense structure, which appeared to form a network of amorphous material, was closely associated with phagocytosed eosinophil granules (Fig. 5A). Immunogold labelled antibodies allowed us to detect an association of the three cationic proteins with this electron-dense network, as illustrated for EPO in Fig. 5B. At higher magnification, this network, always close to phagocytosed eosinophil granules (Fig. 6A), was composed of dark and grey areas. Immunogold labelling showed that MBP was selectively associated with the dark areas (Fig. 6B), while ECP and EPO were located both on the dark and grey areas, as illustrated for ECP in Fig. 6C. The CLC was also phagocytosed in macrophages, but it was detected in areas distinct from altered eosinophil granules and from the network (Fig. 6D). Less than 10% of the

macrophages with eosinophilic proteins in their vacuoles were necrotic.

Discussion

These two cases of EGB had characteristic clinicopathological features. They were found on the skull and rib of young males, producing lytic and well-demarcated defects on radiographs, and not involving the surrounding skin. In both cases, EGB was not a solitary lesion. Case 1 can be classified as a disseminated form of histiocytosis X with pulmonary involvement with the occurrence of a pneumothorax, a frequent complication of pulmonary EGB (Colby and Lombard 1983; McDonnell et al. 1989). In the other case, the two lesions were found in the skull and mandible. Such multiple localizations are not rare (Kaufman et al. 1976) and occur in about 12% of EGBs (Nezelof et al. 1973).

Eosinophils in these two typical cases of EGB were numerous and all of them showed signs of activation. This was also observed both in a bone granuloma and the bone marrow in one case of EGB by Zabucci et al. (1991). The immunostaining of MBP, ECP and EPO – main effectors of eosinophil functions in vitro – allowed us to observe in vivo the possible steps of cationic protein release and to relate them to alterations in eosinophil fine structure. Besides morphological aspects of eosinophil activation in EGB, an interaction between eosinophils and surrounding macrophages could be inferred.

All esoinophils under study showed features of activation; this phenomenon had two different morphological aspects: either cytoplasmic lysis or single granule alteration with occurrence of cytoplasmic tubulovesicular structures.

Approximately 20% of the eosinophils had cytoplasmic lysis; the granules were then free in the extracellular space. The central core alterations of these granules corresponded to massive MBP release. This has been observed in other pathological conditions such as Crohn's disease (Dvorak 1980), bullous pemphigoid (Dvorak et al. 1982), Well's syndrome (Ferrier et al. 1988), and in cell culture (Dvorak et al. 1991). In this type of activation, no degranulation per se occurs; the liberation of granules is the result of cytoplasmic lysis. This can be contrasted to mast cell degranulation, which is not associated with cell death (Asboe-Hansen 1973). Moreover, in our two cases of EGB, immunogold labelling showed that extracellular MBP was reassociated in the close vicinity of the free granules. This suggests a local rather than a systemic effect of ECP. This can also be contrasted to mast cell degranulation, with the preponderant systemic effect of mast cell mediators (Liu et al. 1986).

Most eosinophils in our cases of EGB, as in the case of Zabucchi et al. (1991), were not lytic but only had localized changes of their granules. Immunostaining showed that all three cationic proteins were released from the granules. This is different from the selective release of MBP observed in a case of eosinophilic gastroenteritis by Torpier et al. (1988). With high magnification, some steps in the release of the three granule proteins were detected: localization of the cationic proteins on the ruffled border of altered granules, followed by localization to tubulovesicular structures filling the cytoplasmic areas between the altered granules and the cytoplasmic membrane. In our two cases of EGB, the three cationic proteins appeared to follow the same steps for release. Zabucchi et al. (1991) has shown a similar cytoplasmic pathway, different from granule extrusion, for EPO release in one case of EGB. This closely resembles the "vesicular transport mechanism" described for cutaneous basophil hypersensitivity and might explain the delayed process of release (Capron et al. 1989).

Whatever their mechanism of release, the three cationic proteins showed differences in their extracellular localization and in their distribution in the phagocytic compartments of surrounding macrophages. MBP was easily found at the site of its release from the eosinophil, whereas ECP and EPO were net detected in the intercellular space but were found only in phagolysosomes. Within macrophages, the three cationic proteins were localized near altered granules and were concentrated to the different parts of an electron-dense network. This localization was relatively specific for the cationic proteins since CLC, also detected in macrophages, was never found associated with this electron-dense network. The four phagocytosed proteins were not grossly damaged since they retained their antigenic structures. But they did not exert a cytotoxic effect on host cells (less than 10% of the macrophages containing the labelled proteins were necrotic). Moy et al. (1989) recently demonstrated that eosinophil MBP can activate neutrophils without causing cytolysis. Eosinophils in EGB might activate bone granuloma macrophages in a similar manner. To consider further the role of eosinophils in EGB, it is important to note the concentration of the three cationic proteins was high in macrophages, but totally absent in Langerhans cells. Since lipid-laden macrophages become more numerous as EGB becomes older and more mature (Sparbaro and Francis 1961; Barbey et al. 1982), it is important to understand precisely whether ECP has an effect on the granuloma macrophages, and hence on disease evolution.

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