Br. J. exp. Path. (1981) **62**, 662

ORIGIN OF CRYSTALLOID INCLUSIONS IN MACROPHAGES II: EVIDENCE FOR DERIVATION FROM EOSINOPHIL GRANULOCYTE BREAKDOWN

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Received for publication August 3, 1981

Summary.—Peritoneal macrophages were studied by light and electron microscopy in normal adult mice 24 h after 3 daily injections of preparations of leucocytes and platelets. Crystalloid inclusions, similar to those seen in bone marrow macrophages of normal adult mice and in man, were occasionally observed in peritoneal macrophages after administration of buffy-coat white cells but not after platelets. They were much more frequently seen following the ingestion of eosinophil-rich granulocytes and were almost always associated with secondary lysosomes. Energy-dispersive analysis of X-ray provided further evidence that both crystalloid inclusions and lysosomes originated from the injected granulocytes.

These observations suggest that crystalloid inclusions in marrow macrophages are derived from granulocyte breakdown and that in this respect eosinophil granulocytes are of prime importance.

THE POSSIBILITY that crystalloid inclusions normally present in bone marrow macrophages of adult mice (Hudson, 1968, 1969; Hudson and Shortland 1974, 1980) might be derived from erythrocyte breakdown received little support in an earlier study of peritoneal macrophages following administration of erythrocyte preparations (Ali *et al.*, 1981). In the present investigation, an alternative hypothesis that such inclusions are derived from the breakdown products of ingested granulocytes (Ichikawa and Yoshioka, 1960; Hudson, 1969; Yang, Whest and Nishimura, 1979) has been explored. Peritoneal macrophages were studied following repeated injections of leucocyte, eosinophil-rich granulocyte and platelet suspensions.

MATERIALS AND METHODS

Buffy-coat white cells and platelet-rich plasma were obtained from blood of healthy human donors, and collected into citrate–phosphate–dextrose anticoagulant and centrifuged. The buffy-coat white cells were resuspended in isotonic phosphate-buffered saline (PBS) at pH 7·4. Granulocytes rich in eosinophils were prepared from the blood of a patient in whom over 50% of the leucocytes were eosinophils, the absolute eosinophil count being $7{\cdot}3 \times 10^9$/l. After centifugation at 1000 rev/min for 10 min, packed cells were removed, resuspended in equal volumes of dextran 150 and 3% saline containing EDTA and allowed to sediment. The supernatant was layered on prepared density gradients made up of 2 solutions of colloidal silica (Percoll, Pharmacia Fine Chemicals) and centrifuged at 1000 rev/min for 10 min. The 3 solutions consisted of 27%, 48·6% and 58·5% Percoll in PBS, the granulocytes being collected from the interface between the second and third layers. Microscopy of the material showed that most of the cells were eosinophils, damaged forms and isolated granules being plentiful.

Healthy male white Swiss mice were used, each weighing 30–40 g. Groups of 5 animals received 3 daily i.p. injections of 1 of the 3 cell-preparations or of isotonic PBS. Each injection was given in a volume of 0·5 ml.

Twenty-four hours after the last injection,

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peritoneal cells were obtained, processed and prepared for light and electron microscopy as described in the preceding paper (Ali *et al.*, 1981).

RESULTS

Repeated injections of buffy-coat white cells

On light microscopy, the general appearance of the peritoneal-cell population 24 h after the last of the 3 injections differed little from that of the controls. However, shrunken leucocytes with pyknotic nuclei could be identified either lying free amongst the other peritoneal cells or within the cytoplasm of macrophages. On a few occasions, a single rod-shaped inclusion up to 7 μm in length was noted within a macrophage; such inclusions were never seen in the controls. A few erythrocytes were present.

On electron microscopy, the general features of macrophages were similar to those of control animals (Ali *et al.*, 1981) but they showed rather more phagosomes containing degenerating granulocytes. A crystalloid inclusion was noted on only one occasion. It took the form of an electron-dense rod-shaped profile 1 μm in length; it was surrounded by granular material enclosed within a limiting membrane (Fig. 1).

Repeated injections of eosinophil-rich granulocyte suspensions

On light microscopy, large vacuoles and many round grey bodies were seen within the cytoplasm of the macrophages. Metachromatic mast-cell granules were also present in some macrophages. Dense rod-shaped inclusions 3–8 μm in length were sometimes noted: they were more frequent than in the buffy-coat-injected group, and occasionally more than one could be seen in a single macrophage. They were present in each animal examined.

On electron microscopy degenerating granulocytes could be identified in the macrophage cytoplasm and showed large granules of varying density (Fig. 2). Most of the macrophages contained secondary lysosomes up to 4·5 μm in diameter, corresponding to the grey bodies seen at light-microscope level (Fig. 3). These had a varied content of dense granular material, myelin-like figures, vesicular structures and lipid droplets. Crystalloid inclusions were sometimes present and were almost always found associated with a secondary lysosome (Figs 3, 4, 5). They consisted of electron-dense material with straight sides and blunt, tapering, or occasionally branched ends, the profiles measuring between 2 μm and 7 μm in lengths and 0·1–0·6 μm in width. No special relationship to other cytoplasmic organelles was observed. The crystalloid inclusions were sometimes partially invested by a membrane, which could usually be seen to be continuous with that of the associated secondary lysosome (Fig. 5). Occasionally what appeared to be membrane segments were applied to the surface of the crystalloid inclusion within the secondary lysosome: some of these were seen to be associated with vesicles (Fig. 4). The electron-dense structure of the crystalloid inclusion occasionally displayed small clearly defined electron-lucent areas. An intimate relationship of the crystalloid inclusion with a myelin-like structure within the secondary lysosome was sometimes observed (Fig. 6) and several crystalloid inclusions lying parallel to one another were occasionally noticed. Although the specimens were examined at high magnification, no unequivocal evidence of periodicity was found.

Energy dispersive analysis of X-rays (EDAX) failed to detect iron or zinc in the crystalloid material but showed the presence of silicon in the secondary lysosome and in the crystalloid material (Fig. 7).

Repeated injections of platelet-rich plasma

Under the light microscope, many platelets lay free or in collections in the field. Vacuoles of varying size were frequently noted within macrophage cytoplasm as well as phagocytosed platelets. No example of crystalloid inclusion was encountered. Under the electron micro-

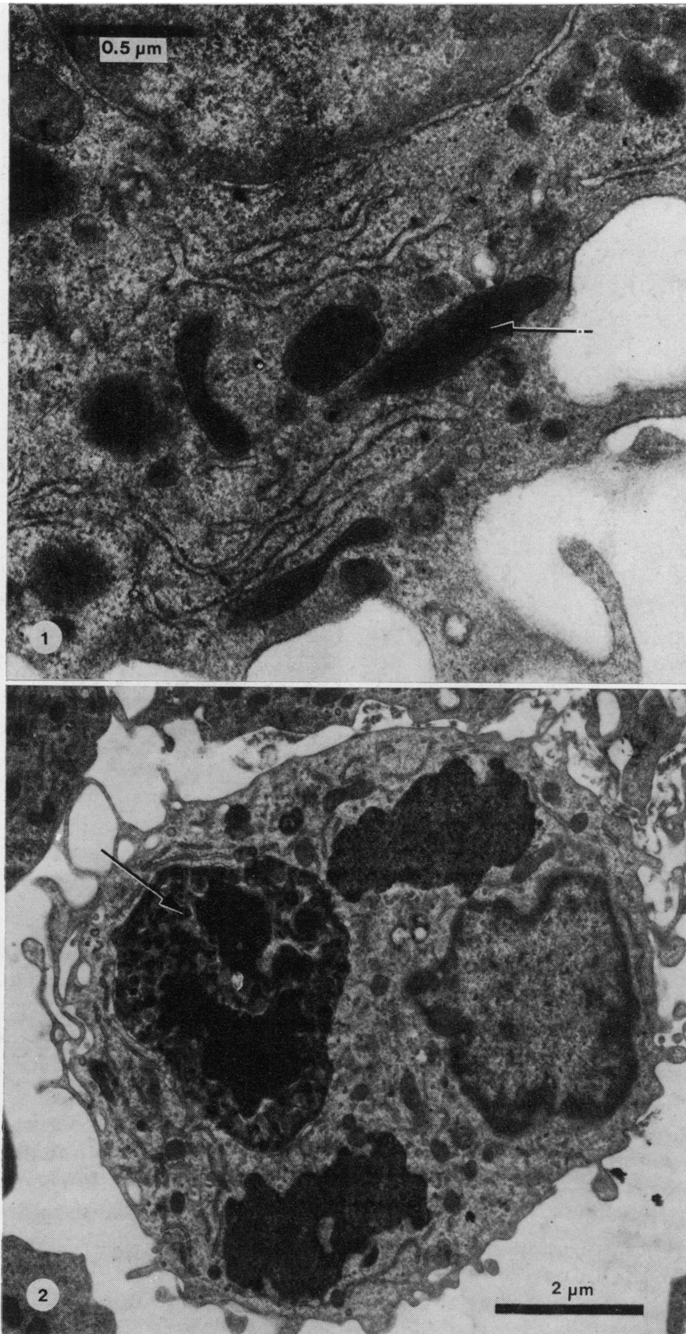
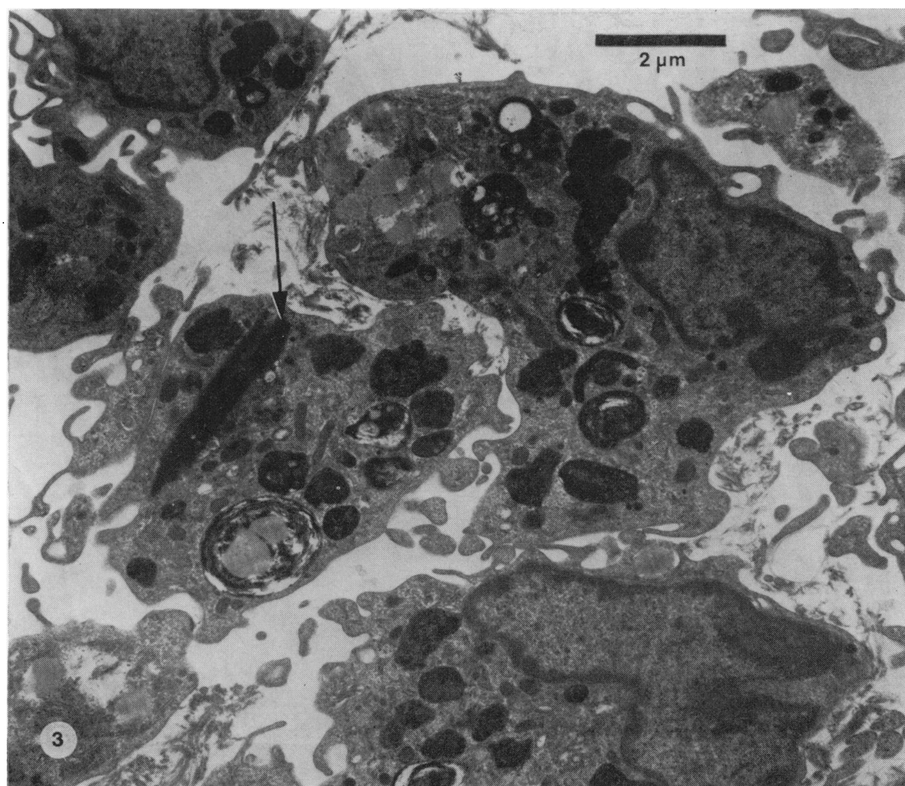


FIG. 1.—Part of peritoneal macrophage from mouse injected with buffy-coat white cells. A small crystalloid inclusion (arrow) is present. It is surrounded by granular material of lower density within a limiting membrane.

FIG. 2.—Peritoneal macrophage following injections of eosinophil-rich granulocyte suspension (ERGS). It contains an ingested cell with numerous granules of varying density (arrow). Two other large granular masses representing cell remains are also present in the cytoplasm.



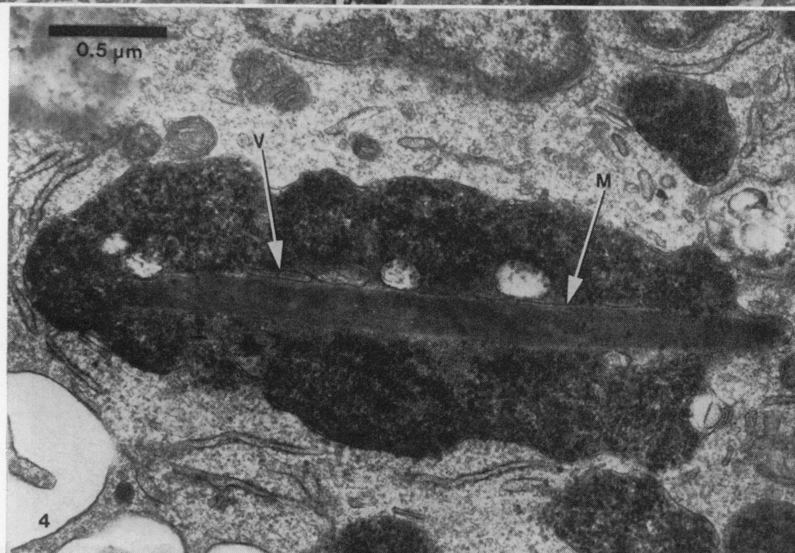
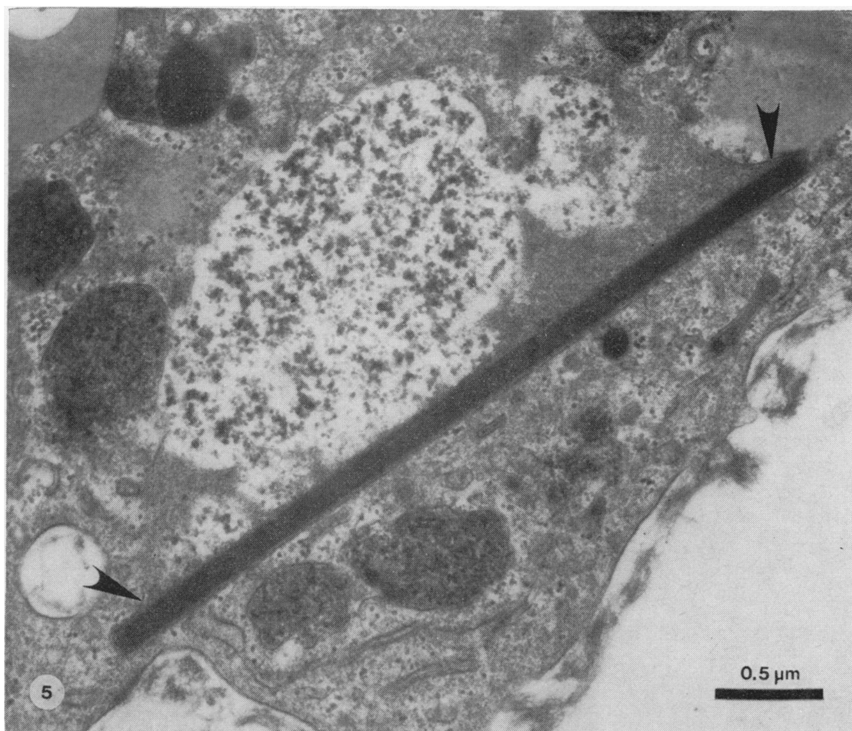


Fig. 3.—Peritoneal macrophages after injections of ERGS. They contain abundant secondary lysosomes with granular material, myelin-like figures and lipid droplets. A crystalloid inclusion which tapers on one aspect and bifurcates on the other is present in one of these cells (arrow).

Fig. 4.—Part of macrophage from peritoneum of ERGS-injected animal. A crystalloid inclusion lies within a membrane-bound granular body (secondary lysosome). Note the presence of interrupted membranes (M) and vesicular structures (V) in relation to the inclusion.



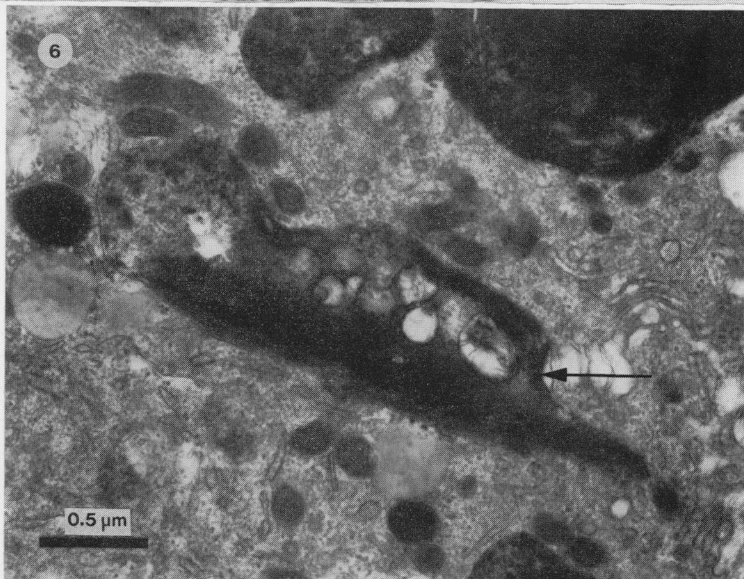


FIG. 5.—Part of a peritoneal macrophage after ERGS injections. A rod-shaped crystalloid inclusion is present. The limiting membrane on the lower aspect of the inclusion is continuous with that of the associated lysosome (arrow heads).

FIG. 6.—Cytoplasm of macrophage after ERGS administration. Crystalloid material is present within a secondary lysosome. It shows a close relation to myelin-like material (arrow).

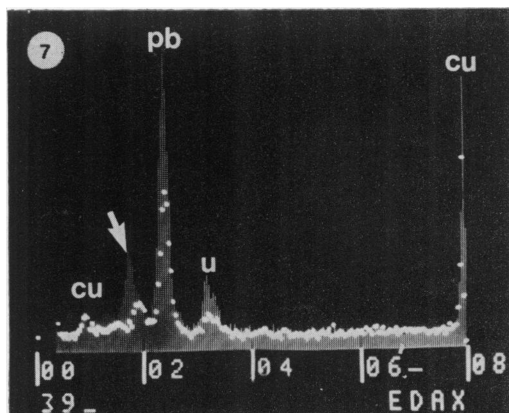


FIG. 7.—Energy dispersive analysis of X-ray of induced crystalloid inclusion in a macrophage following ERGS injections. The main difference from the control area (dots) is a peak indicating the presence of silicon (arrow). The other peaks correspond to the copper of the grid and the heavy metals introduced in preparation, namely osmium, lead and uranium. The peak for osmium of the control area is overlapped by and lies immediately to the right of the silicon peak.

scope, osmiophilic lipid droplets surrounded by an electron-dense granular rim were seen in many macrophages. Myelin-like configurations associated with lipid droplets and phagocytic vacuoles were also noted. No crystalloid inclusions were encountered.

DISCUSSION

The experiments described were designed to test the hypothesis that crystalloid inclusions might be produced in peritoneal macrophages (from which they are normally absent) following the ingestion of large numbers of leucocytes or platelets. For this purpose, macrophages in the marrow and peritoneal cavity have been assumed to have similar properties. The results indicate that crystalloid inclusions were induced in macrophages by both preparations of leucocytes but not by platelets. More inclusions were seen after the injection of a granulocyte fraction in which the majority of cells were eosinophils than after the injection of buffy-coat white cells where the majority of cells were neutrophil granulocytes. Morphology and staining properties of the inclusions were strikingly similar to those seen in marrow macrophages but examples of inclusions over 10 μm in length were not observed. The crystalloid inclusions were almost always associated with secondary lysosomes, lying within their limiting membrane, and this would in itself suggest their derivation from the ingested cells.

The presence of silicon was noted in both secondary lysosomes and crystalloid inclusions following ingestion of the eosinophil-rich granulocyte fraction (Fig. 7) but it was not found elsewhere in the macrophage (cytoplasm or nucleus). Presumably the silicon is derived from the colloidal silica density gradient used in preparation of the fraction. (Silicon is not present in crystalloid inclusions of marrow macrophages.) It is suggested that in these circumstances the silicon might be acting as a granulocyte marker, indicating that both the secondary lysosomes and crystalloid inclusions contain material derived from granulocyte breakdown. The incidence of crystalloid inclusions after the administration of eosinophil-rich fraction strongly suggests that breakdown of eosinophil granulocytes was the significant factor.

The possibility that crystalloid inclusions in marrow macrophages might be derived from granulocyte breakdown has been suggested by a number of previous investigations. Inclusions were first reported present in the marrow of mice with myeloid but not lymphoid leukaemia (Ichikawa and Yoshioka, 1960). In normal mouse marrow, crystalloid inclusions have occasionally been observed within the cytoplasm of degenerating granulocytes which have been phagocytosed by macrophages (Yang *et al.*, 1979).

The possibility that crystalloid inclusions might be derived from eosinophil granulocytes was previously suggested by ourselves (Hudson, 1968) and an example of a crystalloid inclusion within the cytoplasm of an eosinophil granulocyte was illustrated (Hudson, 1969). In view of the known association of eosinophilia and

parasitic infection, it may be relevant that inclusions which appear similar to those induced in the present experiments have been reported in alveolar macrophages of mice infected with *T. canis* (Zyngier and Brockbank, 1974) and of mite-infested monkeys (Leake and Wright, 1976).

Similar inclusions have also been reported in alveolar macrophages of young mice exposed to tobacco smoke and in aged mice under normal laboratory conditions (Matulionis and Traurig, 1977). The significant increase in the frequency of crystalloid material in the bone marrow of normal mice with increasing body wt (Hudson, 1969) could be linked with the significant increase in blood eosinophil counts in these circumstances (Ali, B. A., unpublished data).

The present evidence therefore suggests that crystalloid inclusions in macrophages are derived from eosinophil granulocyte breakdown. This raises the question as to whether they are related in some way to Charcot–Leyden crystals, since eosinophil leucocytes are concerned in the formation of the latter (Welsh, 1959; Archer and Blackwood, 1965; El-Hashimi, 1971; Smith and Forbes, 1972). Charcot–Leyden crystals have a distinctive hexagonal bipyramidal shape, are said to be unique to primate eosinophils (El-Hashimi, 1971) and contain an appreciable quantity of zinc (Buddecke, Esselier and Marti, 1956). The crystalloid inclusions in the present work have a different morphology and fail to show evidence of zinc in their structure on EDAX. Whilst crystalloid inclusions are therefore not Charcot–Leyden crystals, it is possible that they represent an analogous structure in a different species.

We thank Dr R. J. Sokol of the Blood Transfusion Service, Sheffield and Mr Bevington of the Haematology Department, Northern General Hospital, Sheffield for advice and help with the provision of cell samples, and Miss C. Roper for help with the manuscript.

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