Ultrastructural study of human myeloma cells in relation to its function

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SYNOPSIS The ultrastructure of neoplastic plasma cells from a patient with prolonged multiple myeloma was studied in relation to its function, that is, the secretion of immunoglobulin light chain. Peroxidase-labelled antibodies, each monospecific to its immunoglobulin component chain, were used to localize intracellular immunoglobulin within myeloma cells under the electron microscope. By this method, only the κ type light chain was detected within myeloma cells in bone marrow tissue of this patient, indicating that the occurrence of free κ type light chains in serum and urine was due to the cessation of heavy chain synthesis within the myeloma cells. The κ chain was demonstrated as conspicuous electron-dense precipitates in ergastoplasm, its cisternal space, external layer of nuclear membrane, and ribosomes associated with ergastoplasm and nuclear membrane. No immunoglobulin was demonstrated in an atypical Golgi complex, an organelle which is ordinarily engaged in protein synthesis. Numerous crystalline structures and similar inclusion bodies found in myeloma cells appeared to have arisen from the Golgi area, but they did not ever react with the peroxidase label. Discharge of the κ chain from the cell seems to be carried out through cell fragmentation, possibly caused by progressive distension of the ergastoplasmic cavity.

Extensive morphological studies both at light and electron microscope level of human neoplastic plasma cells (myeloma cells) confirmed their marked pleomorphism (Brecher, Tanaka, Malmgren, and Fahey, 1964; Sorenson, 1964; Maldonado, 1966). It ranged from a cell undistinguishable from the normal plasma cell or its precursors to a cell with a clearly abnormal configuration. Pleomorphism seems to extend to subcellular organelles, since various atypical organelles were reported in some myeloma cells (Maldonado, 1966). Furthermore, a variety of cytoplasmic and nuclear inclusion bodies were reported in some of the myeloma cells (Maldonado, 1966b). Little is known, however, of the functional significance of such abnormal subcellular organelles and various inclusion bodies. This is mainly due to the lack of a method

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which allows study of their morphology in relation to their function.

In the present work, myeloma cells found in bone marrow tissue of a myeloma patient were studied by a combination of immunocytochemistry and electron microscopy using peroxidase-labelled antibodies, each monospecific to the immunoglobulin component chain (α , γ , μ , κ , λ) (WHO Bulletin, 1964). The present result strongly suggests that release of the free κ light chain into the serum and urine of this patient was caused by synthesis of the complementary heavy chain within myeloma cells ceasing.

It was also demonstrated that the free κ type light chain is discharged from the cell mainly through cell fragmentation. The significance of various atypical subcellular organelles and inclusion bodies found in myeloma cells is discussed in relation to immunoglobulin production.

Materials and Methods

MYELOMA CELLS

Myeloma tissue was obtained by sternal aspiration from a 50-year-old woman. This patient had multiple myeloma developing over three years. We were the first to report numerous crystalline inclusions in kidney tubules and in the cytoplasm of more than 90% of neoplastic myeloma cells found in the bone marrow. These were never detected in serum or in urine. In the patient's serum, a free light chain of the κ type was detected, and a large amount of Bence-Jones protein, also of the κ type, was excreted in the urine.

ANTIBODY PREPARATIONS

Antisera were produced in goats against human normal IgG, myeloma IgA, and macroglobulin-aemia IgM. Antibodies, each monospecific to the heavy chain class, were specifically purified by using solid immunoadsorbents as described by Takahashi, Yagi, and Pressman (1968). Antibodies, monospecific to the κ or λ type light chains, were also specifically purified by use of immunoadsorbents from rabbit antisera against the Fab fragment of normal human IgG (Takahashi *et al*, 1969). Specifically purified antibodies made monospecific by the addition of soluble serum protein with respect to potency and specificity were greatly to be preferred to the antibody globulin. Details of the purification of antibodies and their characterization have already been described (Takahashi *et al*, 1968, 1969).

CONJUGATION OF ANTIBODY TO PEROXIDASE

Purified antibody was conjugated to horseradish peroxidase¹ according to the method described by Nakane and Pierce (1967). For example, 6.1 mg of purified anti- κ antibody dissolved in 2 ml of 0.5 M carbonate buffer, pH 10.0, was mixed with 7.0 mg of peroxidase, followed by the dropwise addition of 0.25 ml of 0.5% p,p'-difluoro-m,m'-dinitrodiphenyl sulphone² acetone. The reaction was continued at 2°C for six hours. After the reaction, peroxidase-conjugated antibody and unreacted antibody were separated from unreacted peroxidase by 50% ammonium sulphate saturation. Finally, ammonium sulphate was removed by dialysis. The peroxidase-conjugated antibodies thus prepared were tested by immunoelectrophoresis. After electrophoresis was performed, normal human serum or a purified antigen was developed against the corresponding peroxidase-conjugated antibody in the channels. A single and discrete

precipitin arc was always produced. Staining for peroxidase activity by the enzyme substrate, a mixture of 0.075% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in 0.05 M tris-HCl buffer, pH 7.6, showed a brownish reaction product exactly on the precipitin line.

ELECTRON MICROSCOPY

The whole procedure for staining tissue for observation with the electron microscope closely followed the method of Nakane and Pierce (1967). Bone marrow tissue was fixed at 4°C for 40 min using 2% glutaraldehyde as a fixative with gentle agitation. The fixed cell sediment was washed overnight at 4°C with several changes of isotonic phosphate buffer, pH 7.2-7.3 containing 4.5% sucrose. For the reaction, the fixed cell sediment was exposed to peroxidase-conjugated antibody at a concentration of 0.7 to 1.0 mg per ml at 4°C for eight to 12 hours, followed by extensive washings with the same buffer, and finally refixed in 2% glutaraldehyde for one hour to ensure firm binding. The fixative was then washed off with several changes of the phosphate buffer and the cell sediment was placed in Karnovsky's solution without peroxidase (Graham and Karnovsky, 1966; Karnovsky, 1965) at 4°C for one hour, followed by incubation in complete Karnovsky's solution (Graham and Karnovsky, 1966; Karnovsky, 1965) at room temperature for 20 to 30 minutes. The cell sediment was then washed three times with distilled water for 30 min each time, fixed in 2% OsO₄ in phosphate buffer for one hour, then dehydrated and embedded in Epon (Luft, 1961).

The thin sections were cut on an LKB ultratome at a thickness of 500 to 600 Å using a glass knife. They were examined under a Hitachi Hu 11C electron microscope at 75 kV with or without uranyl acetate and lead citrate double staining (Sato, 1967). Controls consisted of myeloma cells exposed in the same manner to peroxidase-conjugated normal rabbit globulin, cells treated with peroxidase-labelled antibody alone, and cells treated with enzyme substrate alone.

Results

A variety of cell types were recognized in myeloma bone marrow tissue by the electron microscope. Neoplastic plasma cells were, however, easily distinguished from other cell types of the non-plasma cell series by such morphological features as a large, rounded nucleus and a quantity of cytoplasm filled with well developed ergastoplasm. When fixed cells were treated with peroxidase-conjugated anti- κ antibody the presence of the immunoglobulin κ chain was shown as discrete electron-dense precipitates within the cytoplasm of more than half of the plasmacytic

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cells (Fig. 1). On the other hand, treating the fixed cell sediment with the peroxidase label of a different antibody specificity failed to reveal any comparable electron-opaque regions in myeloma cells, indicating that the myeloma cell population tested was not producing either immunoglobulin or its heavy chain subunit. Non-plasmacytic cells were never stained by any of the peroxidase-conjugated antibodies except for endogenous peroxidase. Such endogenous peroxidase was prominent within many neutrophil granules encountered in the cytoplasm of granulocytic cells (Fig. 2).

The non-specific adsorption of the peroxidase label can be set aside for several reasons: first, the negative quality of various controls; secondly, the comparison between intact positive cells and intact negative cells; thirdly, the exclusive presence of the κ chain in certain limited regions. The κ chain was localized separately or simultaneously to ergastoplasm, its membrane, external layer of nucleus, and ribosomes lining the ergastoplasmic and nuclear membranes (Figs. 1-3).

In the myeloma cells tested in the present study, ergastoplasm was very prominent, often occupying a large part of the cytoplasm. The reaction of this organelle with peroxidase-conjugated anti- κ antibody indicated that it is actively engaged in the synthesis of the κ light chain. Concerning the development of ergastoplasm, we recognized three cell types among the plasma cell population under investigation. The first cell group resembled the differentiating or immature plasma cells found in spleen and lymph node cells (Bernhard and Granboulan, 1964). In it the ergastoplasm was arranged in concentric rows around the nucleus but ergastoplasmic lamellae were hardly distended (Figs. 1, 2, 6). In the second cell type, ergastoplasmic lamellae were fairly distended, often forming anastomoses with neighbouring ergastoplasm. Their general appearance was like a beehive structure (Fig. 7).

The third cell group was distinguished by extremely dilated ergastoplasmic cavities which invaded almost the entire cytoplasm, causing the cytoplasmic matrix to shrink. The cytoplasmic matrix could hardly be recognized except as a narrow, string-like islet narrowed by the distended ergastoplasmic cavities (Figs. 3, 5, 9). In plasmacytic cells of all three groups, ergastoplasm was always the dominant site of κ chain accumulation (Figs. 1-3). It was noted that even within the same cell, the intensity of the κ chain reaction varied from one ergastoplasm to another. Sometimes positive and negative ergastoplasms were found side by side. In Figs. 1 and 2 are shown electron micrographs of the first cell type which were reacted with peroxidase-conjugated antibody. As shown in Fig. 3, the intracisternal cavity of a myeloma cell of the third cell type was often strongly reactive with the

same enzyme label: the electron density of the ergastoplasm increased after reaction of the cell with peroxidase-conjugated anti- κ antibody. Of interest was the occurrence of the κ chain exclusively within ergastoplasm and the nuclear membrane element, including ribosomes associated with them. We never detected the κ chain leaking into the compressed cytoplasmic matrix.

The Golgi complex was abundant in all the myeloma cells under investigation, so forming the discrete Golgi area. As shown in Fig. 8 Golgi lamellae were deficient in the present myeloma cells. This is somewhat peculiar, because Golgi lamellae are well developed in various tissues engaged in active protein synthesis, such as the pancreatic exocrine cells (Caro, 1961; Caro and Palade, 1964). The main components of the Golgi complex in our myeloma cells were (1) relatively large vacuoles and (2) smaller, more osmophilic vesicles containing electron-dense, homogeneous floccules (Fig. 8). In the Golgi area, we found another kind of vacuole which also contained electron-dense substance. The electron-dense substance in the latter vacuoles occasionally exhibited fine periodic structures which closely resembled larger paracrystalline structures encountered in the cytoplasm of over 90% of myeloma cells in the present study (Fig. 8). This substance was encircled by a single, smooth-faced vacuole (Fig. 8).

The paracrystalline structure, when cut tangentially, exhibited a hexagonal configuration, with 60 to 90 Å between the two structures. A single, smooth-faced membrane, very similar to that of Golgi vacuoles, surrounded one or two such paracrystalline structures (Fig. 11). The evaluation of the results of staining those structures was sometimes difficult, because they were electron-dense even without peroxidase staining. However, the treatment of myeloma cells with peroxidase-conjugated antibody of any specificity never enhanced their electron density, indicating that they did not contain the substance with the antigenicity of immunoglobulin. The membrane of those vacuoles and the space surrounded by it were also unreactive with any peroxidase label (Figs. 1-3).

Relatively large, rounded granules (diameter about 1μ) were found scattered in the cytoplasm unrelated to other cell organelles. A lightly electron-dense, homogeneous substance was contained within the granules. Some of them were reactive with peroxidase-conjugated anti- κ antibody (Fig. 2) but not with the peroxidase label of any other specificity. They resemble zymogen granules in pancreatic exocrine cells (Caro and Palade, 1964). Our staining results suggest that those granules in our myeloma cells are of a similar nature and some of them are engaged in protein secretion.

In general, other cell organelles were scanty and not in a developed form. Smooth-faced ergastoplasm was deficient. Mitochondria, relatively

Fig. 1.

Fig. 1 Electron micrograph of a myeloma cell treated with peroxidase-conjugated anti- κ antibody. A positive reaction is observed as a fine, electron-dense precipitate on ergastoplasm, its membrane, external layer of nuclear envelope. A granulocytic cell (in the upper right) contains neutrophilic granules which are strongly positive due to endogenous peroxidase. Erythrocytes (in the upper part) are also stained by the catalytic activity of haemoglobin. (Lead citrate and uranyl acetate, $\times 10,000$.)

Fig. 2 A part of a myeloma cell treated with peroxidase-conjugated anti- κ antibody but without lead citrate and uranyl acetate double staining. Electron-dense reaction products are recognized simultaneously in ergastoplasmic cavity, ribosomes associated with ergastoplasm and nuclear envelope, and large cytoplasmic granules very similar to zymogen granules. In contrast to an ergastoplasmic cavity, the ground matrix of cytoplasm is definitely negative. Note also the electron lucidity of the Golgi area (G). ($\times 13,000$)

Fig. 2.

Fig. 3.

Fig. 3 Electron micrograph of a myeloma cell of type 3 treated with peroxidase-conjugated anti κ . A strongly positive reaction is noted within the ergastoplasmic membrane (EM). A dilated cisternal cavity occupying a large part of the cytoplasm is stained too. Note that paracrystalline structures (P) are always found outside ergastoplasm. (Lead citrate and uranyl acetate, $\times 10,000$.)

Figs. 4 and 5 *Electron micrographs of myeloma cells in which rupture of the membrane and extreme distension of the cisternal cavity are obvious. (Lead citrate and uranyl acetate, $\times 7,000$ and $11,000$.)*

Fig. 4.

Fig. 6 *A protein of a myeloma cell of type 1 which was not treated with peroxidase-conjugated antibody. The ergastoplasm is arranged in parallel concentric rows. The cisternal cavity is not dilated. Note the electron lucidity of ergastoplasm without peroxidase reaction. Compare with Figure 1. (Uranyl acetate and lead citrate, $\times 25,000$.)*

Fig. 5.

Fig. 7 A myeloma cell of type 2 which was not treated with peroxidase-conjugated antibody. The ergastoplasmic lamellae are arranged in beehive configuration. Note the connecting channel from ergastoplasm to the external layer of nuclear membrane (arrows). Numerous mitochondria are located in the perinuclear region. (Lead citrate and uranyl acetate, $\times 13,500$.)

Fig. 8 High magnification of the Golgi area. The Golgi complex consists of two components: Golgi vacuoles (Va) and Golgi vesicles (Vs). Note the complete absence of lamellar structure in the Golgi area. Some of the peripheral vacuoles contain an electron-dense crystalline structure (arrow) which is very similar to that found in the other part of the cytoplasm. Note also the resemblance between the smooth-faced membrane of those vacuoles and the envelope of larger crystalline inclusions shown in Figure 11.
(Lead citrate and uranyl acetate, $\times 30,000$.)

Fig. 9.

Fig. 10.

Fig. 9 A myeloma cell of type 3 not treated with peroxidase-conjugated antibody. The extremely dilated cisternal cavity occupies the larger part of the cytoplasm. Paracrystalline structures are found always within the narrowed cytoplasmic matrix outside the ergastoplasmic cavity.
(Lead citrate and uranyl acetate, $\times 13,500$.)

Fig. 10 A part of a peculiar myeloma cell containing numerous crystalline structures of irregular configuration. Crystalline structures are highly electron-dense and their fragments are found to scatter around them. In such cells, ergastoplasm, mitochondria, and other cell organelles are very scarce.
(Lead citrate and uranyl acetate, $\times 25,000$.)

Fig. 11 Electron micrograph of crystalline structures cut in longitudinal (L), cross (C), or oblique (O) section. Note that a single, smooth-faced membrane surrounding one or occasionally two crystalline structures is apparently independent of ergastoplasmic membrane. (Lead citrate and uranyl acetate, $\times 45,000$.)

small and packed in an electron-dense matrix, were usually found around the nucleus (Fig. 7). All these cytoplasmic organelles were always unreactive with peroxidase-conjugated antibodies of any specificity. Virus-like particles, as described in the primary and transplanted plasma cell of myeloma cells, were never found within our myeloma cells.

Discussion

Peroxidase-conjugated anti- κ antibody showed the intracellular localization of the κ chain within the cytoplasm of more than 50% of myeloma cells under investigation, whereas peroxidase-conjugated antibodies to other immunoglobulin component chains did not. This clearly indicates that the appearance of a free κ light chain in the serum and urine of this patient was caused by the synthesis of the complementary heavy chain stopping and not by a block in the complementation of an actually synthesized heavy chain to light chain. Such a blockage has been reported in two ascitic lines of mouse plasma cell tumour secreting only free light chain (Schubert and Cohn, 1968).

The general distribution pattern of the κ chain

is very similar to that reported with plasmacytic cells of hyperimmunized animals and murine plasma cell tumours (Rifkind, Osserman, Hsu, and Morgan, 1962; de Petris, Karlsbad, and Pernis, 1963; Leduc, Scott, and Avrameas, 1968, 1969). In our case, positive cells were about 50% of total plasmacytic cells; positive ergastoplasm coexisted with negative ergastoplasm, sometimes side by side. This may suggest the difference in biosynthetic activity among cells or even among ergastoplasms within the same cell.

In positive cells, ergastoplasm was the dominant site of accumulated κ chain. The intracisternal cavity of ergastoplasm was also stained with peroxidase-conjugated anti- κ antibody. This actually contradicts the hypothesis presented by Schubert and Cohn on the assembly of mouse myeloma immunoglobulin (Schubert and Cohn, 1968). They postulated that a specific step is required for the transport of immunoglobulin through endoplasmic reticulum into the cisternal cavity. According to their hypothesis, only a complete molecule of immunoglobulin can be secreted into the cisternal cavity, but subunits of immunoglobulin which are not secreted into the intracisternal cavity are destroyed in the cytoplasm.

Three different types of ergastoplasm were recognized in the present myeloma cell population. They differed from one another in the arrangement of ergastoplasmic membrane and development of cisternal cavity. Our type 1 cells seem to correspond to the lamellar type described by Maldonado, Brown, Bayrd, and Pease (1966), but type 2 and type 3 do not fit any of the six types classified by them. The κ chain was invariably detected in all of the three types, suggesting that cessation of heavy chain synthesis is independent of morphological change of the ergastoplasm.

The numerous cytoplasmic crystals were always unreactive with any peroxidase label. This was a somewhat unexpected observation, because similar paracrystalline structures found in cytoplasm of prolonged multiple myeloma have been identified as gamma globulin by immunoelectrophoresis (Bessis, 1961). The close morphological analysis of crystalline structures in the present myeloma cells suggests that they originated from the Golgi complex rather than ergastoplasm for the following reasons. (1) We could not detect crystalline structures within a distended ergastoplasmic cavity, but exclusively within a narrowed cytoplasmic matrix (Figs. 3, 4, 9, 11). (2) Crystalline structures were always surrounded by a single, smooth membrane which closely resembles that of Golgi vacuoles (Fig. 11). (3) In the Golgi area numerous large granules were observed containing a lightly electron-dense substance in which a periodic fine structure was recognized. The membrane of these granules was undistinguishable from that of Golgi vacuoles (Fig. 8). (4) Peroxidase-conjugated anti-immunoglobulin antibody failed to demonstrate immunoglobulin either in crystalline structures or in the Golgi complex or in inclusion bodies found in the Golgi area. Overall observation suggests that inclusion bodies in the Golgi area are immature forms of larger cytoplasmic crystalline structures with clearer periodicity. The unreactiveness of paracrystalline structures does not necessarily exclude the possibility that they are derived from immunoglobulin or its component subunits (H chain, L chain, or H-L intermediate) (Potter and Kuff, 1964) which were actually synthesized but subsequently destroyed in the cytoplasm. Destruction of such an uncomplemented H chain (Schubert, 1968) or L chain (Schubert and Cohn, 1968) was reported in certain lines of mouse plasma cell tumours.

Using the ferritin antibody method, Rifkind *et al* (1962) identified some globulin not only at the point of transition between the ergastoplasm and the Golgi complex but also within the latter structure itself in mouse plasma cell tumours which were secreting gamma globulin. Using peroxidase as an antigen and a marker, Leduc *et al* (1968) showed that the cisternae of the lamellar portion of the Golgi complex were

always filled with peroxidase-positive material in all antibody-containing plasma cells, whereas the vacuolar components of this apparatus were usually negative. In the present myeloma cells, all elements of the Golgi complex were definitely unreactive with peroxidase-labelled anti-immunoglobulin, thus suggesting that those organelles which are ordinarily engaged in the secretion of immunoglobulin are not active within these myeloma cells.

Regarding the manner in which the immunoglobulin molecules are released in plasma cells, normal and abnormal, two hypotheses have been proposed. The first concept assumes that the Golgi membrane system is involved in packaging protein into vesicles. Those vesicles then transport the protein to the cell surface where it is discharged. The distribution of Golgi vesicles containing electron-dense material both in normal (Bernhard and Gronboulant, 1964) and neoplastic (Dalton, Potter, and Merwin, 1961) plasma cells suggests that such a mechanism may be active in these cells (Bessis, 1961). Reports by Rifkind *et al* (1962) and Leduc *et al* (1968) provide further support for this hypothesis.

Another possible mechanism for the secretion of immunoglobulin by plasma cells was first proposed by Ortega and Mellors (1957) in their immunofluorescence study. This involves the rupture of the cell itself thereby extruding its contents. Thiéry (1960) and Bessis (1961) also described a similar process of cytoplasmic fragmentation, termed 'clasmatosis', in plasma cells of immunized animals and in myeloma cells. The extreme distension of ergastoplasmic cavities in plasma cells containing Russell bodies or protein crystals (Thiéry, 1960) likewise suggests that disintegration of the cell itself may be one mode of release of proteins in plasma cells.

The present result seems to be more consistent with the second hypothesis, since the Golgi complex is not active in the secretion of immunoglobulin, and rupture of cytoplasmic membrane is frequently found in cells with extremely distended ergastoplasmic cavities. However, the presence of small numbers of zymogen-like granules which are reactive with peroxidase-conjugated anti κ suggests that the first mechanism is partly still at work too.

It is fruitless and may be misleading to draw any general conclusion regarding the secretory mechanism of pathological immunoglobulin in myeloma cells from the study of only a single case of myeloma. It is postulated, however, that a similar immunocytochemical analysis of a greater number of tumours and, in particular, a comparison between two types of myeloma cells, one producing a complete molecule of immunoglobulin and the other only its light chain portion, would provide a key to an understanding of the actual nature of atypical subcellular organelles and their relation to defective

immunoglobulin synthesis observed in the second kind of myeloma cell.

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