Intracytoplasmic inclusions in B prolymphocytic leukaemia: ultrastructural, cytochemical, and immunological studies

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SUMMARY The intracytoplasmic inclusions seen in most cells from a patient with B prolymphocytic leukaemia were analysed using both light and electron microscopy. They consisted of a dense homogeneous structure and were surrounded by a membrane, which had no continuity with the Golgi cisternae or the endoplasmic reticulum; some inclusions had a clear association with small lysosomal granules. Immunofluorescence and immunoperoxidase studies using light microscopy failed to elucidate completely the nature of the inclusions, but immunocytochemical reactions performed using electron microscopy suggested an immunoglobulin nature. All inclusions were negative for acid phosphatase and periodic acid Schiff. The nature of the inclusions described in the prolymphocytes of this patient were compared with those previously recorded in B prolymphocytic leukaemia.

Intracytoplasmic inclusions have been described in neoplastic cells from myelomatosis and in lymphocytes from other B cell malignancies, notably B chronic lymphocytic leukaemia, with an incidence of 5–10% of cases. An even higher incidence has been suggested in B prolymphocytic leukaemia. Since our earlier reports of 18 cases we have studied a further 15 cases of B prolymphocytic leukaemia by electron microscopy, and we document here a new case with unusual inclusions within the cytoplasm.

Several types of inclusion have been described in B cell malignancies. One type consists of an amorphous material that lacks periodicity and is contained within an ergastoplasmic membrane. These globular inclusions have the morphological appearance of Russell bodies, and immunofluorescence studies have generally shown them to consist of IgM, although IgG and IgM have also been described. Another type of inclusion is more common and has a periodicity of 60–100 Å, suggestive of a crystalline formation. Pronounced heterogeneity, however, has been noted within this group. Certain inclusions are small needle like structures; others, which have been described as rod shaped or rectangular, are generally large and seen as a single structure within the cell. Usually, these are either associated with the Golgi region or found within strands of the endoplasmic reticulum. Immunofluorescence studies have shown that these inclusions consist of immunoglobulin molecules and are predominantly λ chains, either alone or more commonly in association with IgM or IgA. The origin of the inclusions so far reported in B prolymphocytic leukaemia is not clear. The structures previously documented by our laboratory were long and needle like in one case and displayed periodicity. They also showed a variable degree of acid phosphatase reactivity, which suggested a lysosomal origin. Those described by Kjeldsberg were multiple and rectangular and also displayed periodicity, but no further analysis into their origin or composition was performed.

We describe another case of B chronic prolymphocytic leukaemia in which most cells contained multiple inclusions that differed from those previously described in either B chronic lymphocytic leukaemia or B cell prolymphocytic leukaemia as they lacked periodicity and, morphologically, did not resemble Russell bodies. Cytochemical and immunological studies, using electron microscopy,

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showed that these inclusions comprised molecules of immunoglobulin, which light microscopy suggested to be IgM κ.

**CASE REPORT**

A 73 year old man was asymptomatic until two weeks before admission, when he developed difficulties in micturation, intermittent terminal haematuria, and low abdominal pain. On examination he was pale and tachypnoeic at rest and had echymoses on both lower arms and bilateral crepitations and expiratory wheeze over all zones of the chest. There was no lymphadenopathy or hepatomegaly, but the spleen extended across the midline to the right iliac fossa. Investigations showed haemoglobin concentration to be 73 g/l, platelet count 113 × 10^9/l, and white cell count 262.4 × 10^9/l with 2% granulocytes and 98% prolymphocytes. Urea and electrolyte concentrations and results of liver function tests were normal. Prolymphocytic leukaemia was diagnosed. The patient underwent an uneventful transurethral retrograde prostatectomy and subsequently received a total dose of 320 cGy (rads) to the spleen in 11 courses over five weeks. This treatment reduced the size of the spleen to 1 cm and improved his blood count as follows: haemoglobin concentration 10.3 g/l, platelets 140 × 10^9/l, and white cell count 3.9 × 10^9/l with only 6% prolymphocytes in the differential count.

**Material and methods**

**Light microscopy**

Peripheral blood films were stained with May-Grunwald Giemsa for morphological characterisation of the leukaemia cells. The cytochemical reactions for acid phosphatase and periodic acid Schiff positivity were performed on cyto spin slides made from the mononuclear layer. Peripheral blood mononuclear cells were isolated by Lymphoprep (Nyegaard), washed three times in phosphate buffered saline, and finally resuspended in phosphate buffered saline with 0.2% bovine serum albumin. The following immunological tests were performed: rosette formation with sheep (E rosettes) and mouse (M rosettes) erythrocytes.24 25 Surface immunoglobulin on cell suspensions and cytoplasmic immunoglobulin on cyto spin slides were determined by direct immunofluorescence using fluorescein isothiocyanate conjugated F(ab)₂ antibodies (Behring) against heavy (μ, δ, γ, α) and light (κ, λ) chains after incubation of mononuclear cells at 37°C for two hours to remove cytoplasmic immunoglobulin.

The presence of cytoplasmic immunoglobulin was also studied on cyto spin slides using the method of Stein with monoclonal antibodies anti-μ, γ, α, δ, κ, and λ chains (gift of Dr Ling, Birmingham) as primary reagents and horseradish peroxidase conjugated rabbit antimouse and swine antirabbit (Dakopatts) as a second and third layer, respectively.26 A series of monoclonal antibodies against B cell antigens were tested by conventional indirect immunofluorescence techniques, using a fluorescein isothiocyanate conjugated F(ab)₂ fragment of goat antimouse IgG or IgM (Cappel, Cochranville, Pennsylvania) as a second layer.27

**Electron microscopy**

Mononuclear cells were fixed in 3% glutaraldehyde for 30 minutes, washed in phosphate buffered saline, postfixed in osmium tetroxide, and block stained with 4% uranyl acetate. The cells were pre-embedded in agar, passed through a series of alcohols, and embedded in Araldite. Ultra thin sections were stained with methanolic uranyl acetate and lead citrate and viewed with a Zeiss 10 electron microscope. Acid phosphatase: an adaptation of Gomori's method was used.28 The cells were fixed in 1:5% glutaraldehyde in 0.067 cacodylate buffer pH 7.4 at 4°C, then washed three times in 0.1M cacodylate buffer containing 7.5% sucrose, and incubated for one hour at 37°C in Gomori's medium. Tris maleate buffer containing sodium glycerophosphate as a substrate, and lead nitrate as a coupling agent at pH 5.0.

**Cytoplasmic immunoglobulins**

An adaptation of the method of Newell et al was performed: peripheral blood prolymphocytes displaying typical features of B prolymphocytic leukaemia: a prominent nucleolus and well condensed chromatin. Numerous granules can be seen scattered throughout cytoplasm (arrows) (May-Grunwald Giemsa stain) × 1500.
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Membrane phenotype of peripheral blood cells

<table>
<thead>
<tr>
<th>Test</th>
<th>Reactivity</th>
<th>Reference</th>
<th>Result (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E rosettes</td>
<td>T cells</td>
<td>(24)</td>
<td>2</td>
</tr>
<tr>
<td>Mouse rosettes</td>
<td>B chronic lymphocytic leukaemia</td>
<td>(25)</td>
<td>Negative</td>
</tr>
<tr>
<td>SmIg:</td>
<td>B lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>97 ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgD</td>
<td>93 ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kappa</td>
<td>98 ++</td>
<td></td>
<td></td>
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<tr>
<td>IgA, IgG, lambda</td>
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Cytoplasmic immunoglobulin: B lymphocytes and plasma cells

| IgM; kappa | IgA, IgD, IgG, lambda | 90        | Negative   |

Monoclonal antibodies:

| FMC4        | Class II major histocompatibility complex antigents | (31) | 95         |
| FMC7        | Proportion of normal B and majority of B prolymphocytic leukaemia | (32) | 98         |
| J5          | B cells (gp100)                                             | (33) | 96         |
| OKT10       | Activated B and T cells                                    | (34) | 3          |
| Tac         | IL2 receptor                                              | (35, 36)| 4         |
| UCHT2       | T cells; anti-p67                                          | (37) | Negative   |
| HC1, HC2    | Antihairy cells                                           | (38) | Negative   |

The cells were fixed in 0.1% glutaraldehyde for 15 minutes, washed in phosphate buffered saline and treated with 1% saponin (Sigma) for seven minutes at 55°C. The cells were then washed three times in phosphate buffered saline and incubated with horseradish peroxidase conjugated rabbit antihuman Ig at a concentration of 1:15 for one hour at room temperature. The pellet was then washed and the peroxidase developed using the technique of Graham and Karnovsky. The postfixation was done in 3% glutaraldehyde for 30 minutes and the electron microscopy procedure continued as described above. Test antiserum consisted of horseradish peroxidase conjugated antihuman immunoglobulin (Dakopatts), and horseradish peroxidase conjugated antihuman IgG was used as a control. No counterstaining was performed when viewing the acid phosphatase or cytoplasmic immunoglobulin reactions.

Results

Light microscopy

Most peripheral blood cells had the typical mor-

Fig. 2. Ultrastructure of prolymphocyte with prominent nucleolus (N). Several rod shaped electron dense inclusions may be seen in the cytoplasm (arrows). × 16 000. Sections of Figs. 2–4 are block stained with 4% uranyl acetate.
phology of prolymphocytes with a prominent nucleolus and well condensed chromatin (Fig. 1). Almost all of these cells (90%) had several azurophilic granules in the cytoplasm. These were mainly localised in the perinuclear area, but in some cases they were more widely distributed throughout the cytoplasm.

The Table summarises the immunological phenotype of these cells. Their B cell nature was shown by the strong expression of IgM/IgD κ and class II human leucocyte antigen determinants. Cytoplasmic μ and κ chains were shown in most prolymphocytes, but the analysis of their localisation could be better appreciated in cytocentrifuge slides stained by means of the immunoperoxidase technique. The reaction in these was evenly distributed throughout the cytoplasm with an accentuation in the region of the surface membrane. The azurophilic granules observed in May-Grunwald Giemsa stained slides could not be visualised in these preparations; instead, multiple vacuoles were seen, displaying strong reactivity for both μ and κ chains in their periphery but without any internal reactivity. The localisation and distribution of these vacuoles corresponded to that of the granules seen in the May-Grunwald Giemsa stained slides.

The prolymphocytes were negative for periodic acid Schiff and showed no important acid phosphatase reactivity, although certain cells were weakly positive with a diffuse reaction product. The inclusions failed to stain and showed a weak positivity reaction in their periphery.

**Electron microscopy**

Ninety five per cent of the cells displayed typical features of prolymphocytes with a characteristically prominent, often ring form, nucleolus; 5% showed the features of plasma cells. There was a moderate degree of chromatin condensation with clumping of the heterochromatin in the vicinity of the nucleolus. The Golgi apparatus was poorly developed, and all the cells had only limited profiles of endoplasmic reticulum. Most cells contained a variable number (1–36/cell section) of localised electron dense inclusions, which, invariably, were surrounded by a membrane (Fig. 2). Often several inclusions of different sizes were surrounded by the same membrane, which did not appear to have any association with Golgi cisternae or the endoplasmic reticulum.

The inclusions were seen in close association with small, medium density granules in 40% of cells; in some cells the inclusions seemed to have divided the granules (Fig. 3) The inclusions ranged in size from 0.2 to 0.6 μm in length. They were mainly rectangular or rod shaped, although in some instances the rod shaped inclusions had formed hollow cylinders.

**Fig. 3** (a) Numerous inclusions may be seen contained within a single membrane. Occasionally, an inclusion was seen to bisect a lysosomal granule (arrow). × 30 000. (b) Inclusion distorted into hollow cylinder. × 32 000.

**Fig. 4** High magnification of inclusions. No discernible periodicity was evident. × 73 500.
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Fig. 5 Cell showing acid phosphatase negativity of inclusions (arrows). Note that periphery of inclusions is acid phosphatase positive as is adjacent lysosomal granule (white arrow). × 17 600. Inset shows higher magnification of negative inclusions and acid phosphatase reactivity. × 40 000.

(Fig. 3). No discernible periodicity within any of the inclusions was noted, even at very high magnification (Fig. 4).

The acid phosphatase reaction at electron microscopy confirmed the findings of light microscopy, showing that the inclusions were negative except at their periphery, where they seemed to be slightly positive. The granules that were often seen in association with the inclusions also showed acid phosphatase reactivity (Fig. 5).

Variable reactivity of the inclusions with antihuman polyclonal Ig was observed. In many instances the inclusions and the surrounding membranes were clearly positive (Fig. 6a). In some cells, however, the reactivity was confined to the edge of the inclusion, the core remaining unreactive. Possibly, this partial reactivity may indicate poor penetration of the reagent. No inclusion or membranous structures were seen as positive when antihuman IgG was used as a control (Fig. 6b).

Fig. 6 (a) Cell reactive with antihuman Ig (polyvalent antibody). Note reactivity of cell membrane and inclusions (arrow). Occasional inclusions failed to react (white arrow). × 17 000. Inset shows higher magnification of reactive inclusion. × 40 000.

(b) Cell from control sample using antihuman IgG. No reactivity is apparent either on surface membrane or within inclusion. × 21 000. Inset shows higher magnification of non-reactive inclusion. × 40 000.
Cells from Figs. 5 and 6 were viewed unstained.
Discussion

This report describes the intracytoplasmic inclusions contained within 90% of typical B prolymphocytes from a patient with B prolymphocytic leukaemia. As in most other cases of B prolymphocytic leukaemia, the cells were positive with FMC7 and showed strong expression of SmIg.\(^1\) The reactivity of some cells with OKT10 and antiTac (Table 1) would suggest that a proportion of cells had progressed further in the B differentiation pathway as these markers are associated with plasma cell differentiation\(^2\) and cellular activation.\(^3\)

Ultrastructural analysis confirmed that a small proportion had the morphological characteristics of plasma cells.

The rectangular and rod shaped inclusions were small and composed of a dense homogeneous substance. Periodicity was never observed, even at high magnification. They were contained within a membrane, which had no continuity with either the Golgi apparatus or strands of endoplasmic reticulum. As the inclusions were rectangular, dense, and multiple within each surrounding membrane, they did not have the morphological appearance of Russell bodies and, therefore, could be excluded from the typical globular category. As periodicity was not evident, however, they did not seem to belong to the crystalline category either. The lack of periodicity also indicated that they differed from the needle like inclusions in two cases of B prolymphocytic leukaemia from our laboratory and one reported by Kjeldsberg et al.\(^4\)\(^-\)\(^6\)

Although both the globular and crystalline structures noted in B chronic lymphocytic leukaemia have been shown to consist of immunoglobulin,\(^7\)\(^-\)\(^9\)\(^12\)\(^-\)\(^18\) the nature of the inclusions described in B prolymphocytic leukaemia is uncertain. Costello et al reported an acid phosphatase positivity of some of the inclusions,\(^20\) and Kjeldsberg et al noted that the inclusions were concentrated in the Golgi zone,\(^22\) and on the basis of those findings a lysosomal origin was suggested. Although some of the inclusions seen in the present case were in close association with lysosomal granules and were mainly localised in the perinuclear area, they were, nevertheless, acid phosphatase negative and were also randomly distributed throughout the cell. Thus the lysosomal origin of the inclusions could not be confirmed.

Immunofluorescence studies at light microscopy showed that these B prolymphocytes displayed strong surface reactivity with anti-IgMk. It was, however, difficult to show the presence of cytoplasmic immunoglobulin reliably by this method. Using the more sensitive immunoperoxidase technique,\(^26\) it was clear that though the membranes surrounding the inclusions were positive, the inclusions themselves did not stain. The apparent non-reactivity of the inclusions may have been due to the sensitivity of such structures to certain fixatives.\(^4\) In four other cases of B prolymphocytic leukaemia tested in this laboratory with the immunoperoxidase technique the cytoplasmic reaction was diffuse, with enhancement on the surface membrane, and, unlike the case reported here, vacuoles with peripheral staining were never observed (JVM, unpublished findings).

Immunocytochemical reactions performed at ultrastructural level, however, did confirm the immunoglobulin nature of the inclusions. Reactivity was localised not only in the membrane surrounding the structures but also in the structures themselves. Interestingly, most documented crystalline inclusions contain \(\lambda\) light chain\(^6\)\(^9\)\(^11\)\(^12\)\(^16\)\(^18\) whereas those with globular appearance (type 1) consist of \(\kappa\) chains with either IgM\(^14\)\(^17\) or IgG.\(^13\)

In common with others\(^2\)\(^4\)\(^6\)\(^9\)\(^11\)\(^15\)\(^16\)\(^18\)\(^20\) we observed that the inclusions were negative for periodic acid Schiff. These findings and more recent biosynthetic studies\(^14\) have led to the hypothesis that the immunoglobulin contained within both the crystalline and globular inclusions represents an accumulation of molecules that results from an aberrant secretory mechanism, more specifically from an inadequate addition of carbohydrate.\(^7\)\(^11\)\(^13\)\(^14\)

Although evidence in the present case is far from conclusive, we are tempted to speculate that the inclusions described here may be a result of a similar kind of defect.

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References

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