Biosynthesis and characterisation of IgM λ in a case of chronic lymphocytic leukaemia with intracellular immunoglobulin inclusions

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SUMMARY Lymphocytes from a patient with chronic lymphocytic leukaemia were found to contain crystalline inclusions in the cytoplasm. These crystals were characterised as IgM λ immunoglobulin. The cells did not have detectable surface immunoglobulin. No paraprotein was detected in the serum and no excess of immunoglobulin was detected in the urine. The cells incorporated radioactive leucine into μ and λ chains during an 18-hour culture. Studies of labelled lysate immunoglobulin revealed a molar excess of heavy chain over light chain while in contrast free light chain was the only detectable secreted immunoglobulin product during the culture period. These findings are discussed in relation to immunoglobulin synthesis by normal and other neoplastic lymphocyte preparations.

The lymphocytes of patients with chronic lymphocytic leukaemia (CLL) may contain crystals of immunoglobulin in their cytoplasm (Clark *et al.*, 1972; Hurez *et al.*, 1972; Cawley *et al.*, 1973). It has been suggested (Cawley *et al.*, 1976) that these crystals can be found in the lymphocytes of about 5% of patients with CLL. This report describes a case of CLL where more than half the lymphocytes contained IgM λ inclusions.

In previous case reports the cells with intracellular IgM λ type crystals (Clark *et al.*, 1972; Hurez *et al.*, 1972; Preud'homme and Seligmann, 1972; Cawley *et al.*, 1976) expressed the same immunoglobulin on the cell surface membrane. The failure to express surface immunoglobulin has been reported in one case with crystalline cytoplasmic inclusions where the cells contained IgA λ (Cawley *et al.*, 1973). The case described in this report did not have detectable surface IgM λ .

Clinical data

A retired carpenter aged 82 presented in September 1976 with a history of necrotic ulcers on the lips, neck, and chest for two months. He had had a

similar lesion on the scrotum five months previously which had healed spontaneously. Apart from a prostatectomy in 1973 for benign prostatic hypertrophy he had had no serious illnesses.

On examination there was axillary and inguinal lymphadenopathy and the liver and spleen were both moderately enlarged. Biopsy of the ulcers showed severe vasculitis but no leukaemic infiltration. The haemoglobin was 11.5 g/dl, total white count $62.0 \times 10^9/l$ with 1% neutrophils and 99% mature lymphocytes. More than 55% of the lymphocytes contained cytoplasmic inclusions. Platelets were $140 \times 10^9/l$. Total protein was 60 g/l and albumin 41 g/l. There was a decrease of all the gamma-globulins: IgG 2.8 g/l, IgM < 0.4 g/dl, and IgA < 0.4 g/l.

He was treated with prednisone, 15 mg, and chlorambucil, 5 mg, daily, and on this treatment the ulcers improved and the lymph nodes became smaller. The spleen remained unchanged in size.

One year later the haemoglobin is 12.4 g/dl, total white count $18.7 \times 10^9/l$, neutrophils 7%, monocytes 1%, and mature lymphocytes 92%. The intracellular crystalline inclusions detected at presentation are still present. The ulcers have healed, the lymph nodes are not enlarged, but the spleen is still the same size.

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Material and methods

CYTOLOGICAL AND CYTOCHEMICAL STUDIES

In addition to staining with Leishman's stain, smears of whole blood were stained with peroxidase, Sudan black, periodic acid Schiff, and alkaline phosphatase stains using routine haematological cytochemical methods (Hayhoe and Flemans, 1969). Pure lymphocyte preparations free of erythrocytes were prepared from heparinised blood by triosil-ficoll gradient sedimentation (Thorsby and Bratlie, 1970). The cells were washed three times with Modified Eagles Medium and subsequently used for immunofluorescent studies. Cytocentrifuge preparations were also stained for protein using the Ninhydrin-Schiff method (Yasuma and Ichikawa, 1953).

ELECTRON MICROSCOPY

Lymphocytes were separated from heparinised whole blood by dextran sedimentation, fixed in 1.5% glutaraldehyde, and processed for electron microscopy as previously described (Cawley and Hayhoe, 1973).

IMMUNOFLUORESCENT STAINING

Membrane fluorescence was performed on washed lymphocytes after incubation for one hour at 37°C by the direct method using fluorescein-labelled rabbit and sheep antisera against μ , γ , α , and δ heavy chains and k and λ light chains; fluorescein-labelled rabbit and sheep normal sera were included in all experiments. The preparations were stained for 20 minutes on ice and washed three times with cold phosphate buffered saline (PBS) before resuspension in 50% glycerol in saline. The antisera were raised by immunisation with myeloma and/or pooled normal purified immunoglobulin as antigen, and were then absorbed with purified immunoglobulin coupled to Sepharose 4B to remove unwanted cross-activities. All antisera were checked for specificity by their ability to stain purified myeloma proteins coupled to Sepharose G-25.

Cytoplasmic immunofluorescence preparations of washed lymphocytes were fixed overnight in dry acetone at -20°C and stained by the direct method. Controls using normal rabbit or sheep sera were included in all experiments.

Fluorescent-stained fixed smears and viable cell suspensions were mounted in 1:1 glycerol solution and examined under a Zeiss epiluminescent microscope fitted with an Osram HPO 200 mercury vapour lamp.

IMMUNOGLOBULIN STUDIES

Serum was assayed for immunoglobulin levels using

a laser nephelometer (Travenol Labs Ltd, UK) and was electrophoresed on plastic-backed cellulose acetate plates (Millipore UK Ltd). An aliquot of a 24-hour urine was concentrated $\times 200$ and analysed by electrophoresis. Immuno-electrophoresis was carried out using preformed agar plates (Millipore UK Ltd) and antiserum to IgG, A, M, D heavy chains, and k and λ light chains.

ROSETTE TESTS

Tests for rosette formation with ox and sheep cells prepared using the method of Smith and Haegert (1974) were carried out to identify E, Fc, and C3 receptors on the cell surface.

IMMUNOGLOBULIN SYNTHESIS

Biosynthetic labelling techniques and the subsequent characterisation and quantification of labelled immunoglobulin have been described in detail elsewhere (Gordon *et al.*, 1978). Briefly, cells at 5×10^6 /ml were incubated in leucine-free medium containing ^3H -leucine at 25 $\mu\text{Ci}/\text{ml}$ for 18 hours at 37°C. Cells were separated from the supernatants by centrifugation and lysed with PBS containing detergent NP₄₀ and proteolytic inhibitors. Both the cell lysates and supernatants were spun at 35 000 g to remove cell debris and dialysed exhaustively against PBS.

The radioactivity incorporated in all macromolecules was determined by precipitation with 10% trichloroacetic acid (TCA). Labelled immunoglobulin was precipitated using a sandwich technique with sheep antiserum specific for human immunoglobulin as the first antibody and rabbit antiserum with activity to sheep immunoglobulin as the second antibody. Normal sheep serum was used as the first antibody in control precipitations. These immunoglobulin precipitates were washed three times with cold PBS before counting or preparation for gel analysis. Reduced and alkylated immunoglobulin precipitates were analysed on 9.5 cm long 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis with radiolabelled myeloma IgG and IgM markers.

Results

CYTOCHEMICAL STUDIES

In Romanowsky preparations the crystals appeared as large, unstained, rod-like shapes in about 55% of the lymphocytes (Fig. 1). The inclusions did not stain with routine haematological cytochemical stains (peroxidase, PAS, and alkaline phosphatase) but reacted positively with the Ninhydrin-Schiff method.

Fig. 1 Romanowsky preparations showing crystalline inclusions. $\times 40$

FLUORESCENT STUDIES

The crystals were shown to be IgM lambda in nature (Fig. 2). The crystals were large and rod-shaped, and some cells had more than one in the cytoplasm. No surface immunoglobulins were detected on the surface of the lymphocytes. The incidence of the crystals in the fluorescent preparations was about 60%; this proportion is somewhat higher than that seen in the Romanowsky preparations and is consistent with previous data.

SERUM ELECTROPHORESIS

No paraproteins were detected in the serum but the gammaglobulin was markedly depressed, and the levels of IgM, IgA, and IgG were 2.8, 0.4, and 0.4 g/l respectively. Normal levels for this laboratory are IgG 7.2-13.0, IgA 1.0-3.6, and IgM 0.5-1.5 g/l. No excess of immunoglobulin fragments was found in the urine.

ELECTRON MICROSCOPY STUDIES

The majority of cells contained only one crystal, although two or more were seen in some cells (Fig. 3).

IMMUNOGLOBULIN SYNTHESIS STUDIES

During an 18-hour culture period the patient's cells synthesised and secreted immunoglobulin precipitable with polyvalent antiserum to human

immunoglobulin (Table 1). The amount of immunoglobulin synthesised expressed as a percentage of the total protein produced was comparable to other cases of CLL but less than normal peripheral blood cultures (Table 2).

Antiserum for light chain precipitated all the labelled supernatant immunoglobulin from the patient while no significant counts above control were precipitated with K-light chain specific antiserum (Table 1). Gel electrophoresis of the reduced and alkylated supernatant immunoglobulin revealed a light chain peak but no detectable heavy chain peak. This contrasted with a normal peripheral blood culture supernatant which showed significant peaks in both heavy and light chain regions (Fig. 4). Gel analysis of the patient's lysate revealed a light chain peak and a heavy chain peak with a mobility similar to myeloma μ chain (Fig. 4). A third peak migrating just faster than the γ chain marker was present in both specific and control lysate precipitations from normal peripheral blood, the patient's blood, and other CLL cultures. From the counts associated with the heavy and light chain peaks on gel analysis, the molar ratio of the two chains can be calculated: 1.0 would indicate a balanced synthesis. Consistent with other cases of CLL, free light chain was the only detectable secreted immunoglobulin product from the patient (Table 2). Labelled lysate immuno-

Fig. 2 Fluorescent preparations. Cells stained with fluorescein conjugated anti-human λ antisera examined under ultraviolet light. Similar results obtained with anti-human μ . $\times 100$

Table 1 Immunoglobulin synthesis by patient's cells

Culture fraction	cpm of ^3H -radioactivity precipitated by*:				
	TCA (total protein)	Polyvalent Ig antiserum	Normal sheep serum (control)	Anti- λ chain antiserum	Anti- γ chain antiserum
Supernatant	140 000	4000	1200	4300	1400
Lysate	1 344 000	8800	3200	nd	nd

*Results given are cpm of ^3H -radioactivity incorporated per 10^7 cells.

nd = not determined.

All results are the mean of three experiments.

Table 2 Heavy and light chain synthesis by normal and neoplastic cells

Sample	Supernatant Ig		Lysate Ig	
	Ig as % of total protein synthesised	Light/Heavy* (molar ratio)	Ig as % of total protein synthesised	Light/Heavy (molar ratio)
PUT	0.2	LC only†	0.4	0.7
'Typical' CLL‡	0.4	LC only	0.4	5.3
(mean of eight cases)	(range 0.1-0.7)		(range 0.1-0.9)	(range 1.2-13.0)
Normal peripheral blood	0.9	1.1	0.6	1.1
(mean of five samples)	(range 0.2-1.5)	(range 1.0-1.3)	(range 0.3-0.7)	(range 1.0-1.2)

*Molar ratios calculated on the basis of 17 leucine residues in light chain and 38 residues in heavy chain.

†Labelled light chain only detected on gel electrophoresis.

‡Cases where the neoplastic cells did not have detectable cytoplasmic Ig.

Fig. 3(a)

globulin from the patient showed a molar excess of heavy chain; all other cases of CLL revealed an intracellular light chain excess. Normal peripheral blood lymphocytes showed either a balanced synthesis or only a slight light chain excess (Table 2).

Discussion

Crystalline inclusions in CLL have been reported previously (Bessis, 1951; Bernard *et al.*, 1959;

De Man and Meiners, 1962; Hurez *et al.*, 1972; Cawley *et al.*, 1973). It is interesting to note that in the previous cases with IgM lambda crystals (Clark *et al.*, 1972; Hurez *et al.*, 1972; Preud'homme and Seligmann, 1972; Cawley *et al.*, 1976) the immunoglobulin of the crystals was expressed at the surface of the cell. In our case no surface immunoglobulin could be detected. The significance of this result is not clear, nor indeed is the clinical significance of the crystals.

Fig. 3(b)

Fig. 3 Electron micrograph of cells. Note size of inclusion: (a) $\times 33\,750$; (b) $\times 41\,600$

The light chain specificity of the labelled immunoglobulin in the culture supernatant was consistent with the immunofluorescence data and provided good evidence for its neoplastic cellular origin. The rate of the immunoglobulin synthesis and secretion was comparable to that of other cases of CLL and at first analysis revealed no indication of a secretory block. Consistent with recent reports (Gordon *et al.*, 1978; Maino *et al.*, 1977) the patient's lymphocytes shared with other CLL cells the capacity to synthesise and secrete free light chain *in vitro*. However, a unique feature of this case was the accumulation of an intracellular, labelled, heavy chain excess which was not secreted during the culture period.

It has been suggested that the glycosylation of the

polypeptide chain may be an essential element in the secretion of some immunoglobulin molecules (Melchers and Andersson, 1973), and a defect in this mechanism might explain the lesion in this case. Negative PAS staining, suggesting an absence of glycoprotein within the inclusions, would be consistent with such a defect. However, the process of carbohydrate addition may be operative in these cells as the intracellular heavy chain had a mobility on sodium dodecyl sulphate gels similar to that of the myeloma μ chain; any significant deletion in either the polypeptide chain or the number of carbohydrate residues would presumably have resulted in an apparently lower molecular weight.

However, Nies *et al.* (1976) have postulated that

Fig. 4 7.5% Polyacrylamide gel electrophoresis of reduced and alkylated immunoglobulin from peripheral blood culture supernatant and lysates. The figure illustrates a gel profile from (a) normal peripheral blood, and (b) the patient. The positions of myeloma μ , γ , and light chain (L) markers are shown: specific precipitation: — non-specific precipitation.

an aberrant or even excess addition of carbohydrate might be responsible for the inability of some cells to secrete immunoglobulin and that this could result in the subsequent formation of intracytoplasmic inclusions. As the addition of carbohydrate does not appear to be a necessary prerequisite for light chain secretion (Eagon and Heath, 1977), the capacity of our patient's cells actively to secrete free light chain but not heavy chain would be in accord with such a defect.

The third peak in both specific and non-specific precipitations of the lysate has been reported previously (Gordon *et al.*, 1977) and may relate to the Fc receptor (Vitetta *et al.*, 1974).

The absence of an abnormal μ chain component in the serum or urine was consistent with the biosynthetic studies while the inability to detect Bence-Jones protein in $\times 200$ concentrated urine by electrophoresis probably reflects the increased sensitivity

of the *in vitro* biosynthetic studies. Our inability to detect surface IgM is consistent with a defective immunoglobulin transport mechanism in the cell and suggests that the nature of the lesion may be more primitive in our patient's cells than in those cases expressing membrane immunoglobulin.

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