Intracellular inclusion bodies in 14 patients with B cell lymphoproliferative disorders

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SUMMARY Two types of intracytoplasmic inclusion were detected by immunofluorescence microscopy in 12 patients with chronic lymphocytic leukaemia and two patients with a leukaemic phase of well differentiated lymphocytic lymphoma. Further analysis with light- and electron microscopy, showed that most inclusion bodies were rod-like crystalline structures. However, in three patients they consisted of amorphous vesicular precipitates.

Immunological studies revealed the presence of immunoglobulins of the same class and type at the cell surface as well as in the inclusion bodies. The monoclonal immunoglobulins were all of lambda type except in two cases. The origin of immunoglobulin inclusion bodies in B cell malignancies is discussed in relation to published data and our own observation in one patient followed during treatment.

B chronic lymphocytic leukaemia (CLL) and lymphocytic lymphomas can in general be regarded as a neoplastic proliferation of B lymphocytes "frozen" at an immature maturation stage, characterised by the presence of surface immunoglobulins only.¹

As B lymphocytes, early in their differentiation, get committed to the synthesis of an Ig with an unique V_H-V_L region—ie idiotype—associated with a single light chain isotype,² a malignant B cell transformation will give rise to a malignant clone of cells each carrying identical properties when compared to the original transformed cell.

It follows that in one patient all malignant B cells synthesise identical Ig molecules. Although multiple heavy chain classes can be expressed on the same cell, the exclusive expression on one light chain isotype can be used as a criterion for monoclonality, while the idiotype has to be regarded as a tumour specific marker.³

Several reports on patients with B-CLL have indicated that the malignant lymphocytes of some CLL patients manifest intracytoplasmic inclusion bodies consisting of monoclonal immunoglobulins. These intracytoplasmic inclusions have been ob-

served in about 3-7% of all CLL patients,⁴⁻⁷ depending on the technique used. Immunofluorescence microscopy seems the most sensitive method for their detection. Most of these inclusions, are of IgM lambda nature,^{4,12} but isolated cases are reported expressing IgM kappa,¹³ IgA lambda,⁵⁻¹⁴ IgG kappa¹⁵ or lambda light chain only.¹⁶ In each case the surface immunoglobulin is identical to the one found in the inclusion bodies. Similar inclusion bodies have been demonstrated in lymphoproliferative disorders other than CLL, such as mixed lymphocytic-histiocytic lymphoma,¹⁷ immunoblastic lymphoma¹⁸ and multiple myeloma.¹⁹

In our laboratory B-lymphoproliferative diseases are routinely investigated for the presence of intracellular immunoglobulins by immunofluorescence microscopy. In this way intracellular inclusions were found in 14 patients. Two different types of intracytoplasmic inclusions were observed. In most of the 14 patients the inclusions had a rod-like structure, in only three patients we observed a globular structure. On no occasion were both types of inclusions present simultaneously in the same patient. The inclusion bodies of seven patients were studied by electron microscopy. The presence of Ig containing crystals was followed in one patient during chlorambucil treatment and will be discussed in more detail.

Material and methods

PATIENTS

The cytoplasmic inclusions were detected by a systematic examination of the peripheral blood of patients with a B-lymphoproliferative disorder, using anti-immunoglobulin sera and cytoplasmic immunofluorescence microscopy. Further immunological and morphological studies were then carried out. The diagnosis of chronic lymphocytic leukaemia in 12 patients with intracytoplasmic inclusion bodies was made on the basis of chronic peripheral lymphocytosis ($>4000 \text{ mm}^3$) combined with infiltration of the bone marrow ($>40\%$ lymphocytes). Patients were allocated to one of the five anatomicoclinical stages as proposed by Binet *et al.*:²⁰ five patients belonged to stage 1 and six patients to stage 3, whereas the initial clinical findings were not available in one patient. In two other patients, intracytoplasmic inclusion bodies were seen during the leukaemic phase developing several years after the initial diagnosis of well differentiated lymphocytic lymphoma (WDLL).

IMMUNOFLUORESCENCE STUDIES

Surface immunoglobulin (sIg)

Peripheral blood mononuclear cells are isolated by Ficoll-Hypaque centrifugation of venous EDTA blood. Cells recovered from the interface are washed three times in phosphate-buffered saline (PBS), containing 1% bovine serum albumin (PBS-BSA) at pH 7.2. A suspension of 30×10^6 cells/ μl is made. To allow shedding of cytophilic Ig, the mononuclear cell suspension is incubated for two hours at 37°C in a 5% BSA solution. In order to inactivate the Fc receptors of the so-called undefined mononuclear cells prefixation with paraformaldehyde 0.04% is performed for 10 min at room temperature.^{21, 22} Surface immunoglobulin positive lymphocytes in the mononuclear cell suspension are assessed using monospecific antihuman Ig antisera conjugated to FITC or TRITC. Detailed specifications have been published.²³ After a thorough washing procedure, the cells are mounted on microscopical slides in buffered glycerol and sealed. All preparations are viewed using a Leitz Dialux® Ploem Opak fluorescence microscope, equipped for the two wavelengths method with simultaneous use of phase contrast optics for the morphological identification of the cells. The percentage of lymphocytes having sIg determinants is determined.

Cytoplasmic immunoglobulins (cIg)

A washed suspension of approximately 1.5×10^6 peripheral blood mononuclear cells per ml is sedimented by a cytocentrifuge as described earlier.²⁴ Staining is performed by incubation of the fixed and

washed slides with the same conjugated antisera as used for sIg staining.

The cIg positive cells are counted. Their number is expressed as a percentage of the lymphocytes present in the preparations.

LIGHT MICROSCOPY

Blood smears were stained with May-Grünwald-Giemsa and periodic acid-Schiff reagent (PAS).

ELECTRON MICROSCOPY

Lymphocyte preparations are fixed at room temperature for one hour in 2.5% glutaraldehyde in 0.075 M *s*-collidine buffer (pH 7.2). After overnight rinsing with 0.2 M *s*-collidine buffer, the cells are fixed for one hour in 1% unbuffered osmium tetroxide. Prior to dehydration they are stained with 0.5% uranyl acetate at pH 5.3 for one hour. They are embedded in Spurr resin. Ultrathin sections are contrasted with lead citrate and examined with transmission electron microscopy.

Results

Fourteen patients, expressing intracellular inclusion bodies detected by cytoplasmic immunofluorescence microscopy, were studied. The surface immunoglobulins (sIg) and the immunoglobulin content of the intracellular inclusions in 14 patients are summarised in the Table. In two cases (Cre and Sch) the sIg expression was not determined, in one case (Vho) no sIg was detectable. In 10 of the remaining 11 patients, the sIg's belonged to the lambda light chain type and the kappa light chain type was only seen once. The IgM heavy chain was seen in 10 cases and in two of them IgD was expressed simultaneously. One patient carried sIgA only. In all cases the fluorescence intensity of the surface immunoglobulin was faint and often only a few sIg positive cells could be visualised. In contrast, the inclusions exhibited a bright fluorescence staining while the surrounding cytoplasm was negative. It is important to note that immunoglobulin expression of the inclusion bodies was identical to the one found at the surface level, except in two cases (Vdu and Vke) where no IgD was found in the crystals. Finally, one patient (Vho) presented with IgG kappa inclusions without visible surface immunoglobulins. Details of this exceptional case, will be published elsewhere.

Often multiple inclusion bodies were noticed in one lymphocyte. The number of lymphocytes containing inclusions was always lower than the amount of sIg bearing lymphocytes, with again case Vho as the exception. It is interesting to note that the percentage of inclusion containing lymphocytes ranged from 1 to 54%. The morphological appear-

Morphological and immunofluorescent study of peripheral blood lymphocytes

Patients	Sex	Age(yr)	Immunofluorescence			
			Morphology	% inclusion positive lymphocytes	Inclusion Ig type	Predominant surface Ig
Cla	M	72	Rod-like	54	IgA- λ	IgA- λ
Vha	F	83	"	6	IgM- λ	IgM- λ
Cre	M	68	"	15	IgM- λ	Not done
Sch	M	53	"	3	IgM- λ	Not done
Buy	M	66	"	3	IgM- λ	IgM- λ
Lau	M	60	"	1	IgM- λ	IgM- λ
Vho	F	70	"	40	IgG- λ	Absent
Vdu	M	48	Globular	3	IgM- λ	IgM-D- λ
Gee	M	49	"	8	IgM- λ	IgM- λ
Goo	F	82	"	41	IgM- λ	IgM- λ
Vke	M	73	Rod-like	3, 5	IgM- λ	IgM-D- λ
Bie	F	56	"	44	IgM- κ	IgM- κ
Dvo	M	68	"	3	IgM- λ	IgM- λ
Dwi	F	56	"	4	IgM- λ	IgM- λ

ance of most inclusion bodies was needle-like, while in three cases (Vdu, Gee and Goo) they had rather a globular structure. Both types of inclusions were never seen simultaneously in one patient. In May-Grünwald-Giemsa stained peripheral blood films intracellular inclusion bodies were found in the lymphocytes of six out of the 14 patients (Cla, Cre, Vho, Gee, Goo, Bie). In most cases they appeared as large, unstained rectangular structures (Fig. 1a), while in others they presented as small globular vesicles (Fig. 1b). Some lymphocytes contained multiple inclusions of the same type. The inclusions were PAS-negative. In seven patients the ultrastructural features of these inclusion bodies were studied by electron microscopy. The morphological characteristics of medium-sized lymphocytes with a relative abundance of free ribosomes were confirmed. As already suspected by light and immunofluorescence microscopy, two types of inclusions could be distinguished. In cases Cla, Vha, Cre and Vho the inclusions appeared as electron dense crystalline structures, consisting of laminar material on longitudinal section, with a periodicity of 40 to 60 Å,

surrounded by rough endoplasmic reticulum (Figs. 2, 3). The lymphocytes of cases Vdu, Gee and Goo had pronounced dilatation of endoplasmic reticulum,

Fig. 1 (a) and (b) Light microscopic aspect of the two types of intracytoplasmic inclusions. In May-Grünwald-Giemsa stained peripheral blood smears the intracytoplasmic inclusions appeared as (a) unstained rod-like (arrow) or (b) globular structures (arrow) $\times 1140$.

Figs. 2, 3, 4, 5 Electron microscopic aspect of the two types of intracytoplasmic inclusions. The rod-like inclusions (Figs. 2, 3 arrow) showed a crystalline structure surrounded by endoplasmic reticulum, while the globular inclusions were formed by dilated strands of rough endoplasmic reticulum containing an amorphous material (Figs. 4, 5 arrow).

Fig. 3

Fig. 4

Fig. 5

containing an amorphous material without evident periodicity (Figs. 4, 5).

To evaluate the relation between inclusion positive lymphocytes and surface immunoglobulin positive lymphocytes, both cell subtypes were examined several times during the course of a chlorambucil treatment in one patient (Cla). Fig. 6 indicates that before treatment the peripheral blood lymphocyte count was 58000 mm³, of which 55% expressed sIgA-lambda and 50% had crystalline inclusions. After treatment the lymphocyte count was near to normal. However, during a relapse the sIg positive lymphocytes increased again up to 43% while the relative numbers of crystal positive cells remained very low.

Discussion

This report deals with 14 cases of chronic lymphocytic leukaemia (CLL) and well differentiated lymphocytic lymphoma (WDLL) in which the malignant cells were shown to contain inclusion bodies consisting of immunoglobulins as demonstrated by immunofluorescence and electron microscopy.

In the last decade a number of reports have appeared describing this anomaly^{5-19 25-28} in B cell lymphoproliferative diseases of which CLL forms the large majority. The frequency of these inclusion bodies depends on the morphological techniques used, but can be estimated to be about 5% of all CLL patients. In this context, the intracytoplasmic immunofluorescence staining seems to be the most sensitive method. This technique confirmed the Ig

Fig. 6 Evolution of lymphocytosis during treatment of patient (Cla): surface immunoglobulin positive (sIg) and crystalline immunoglobulin containing (crIg) lymphocytes are plotted in absolute number (mm^3); Leukeran (■) was given on two occasions.

content with a monoclonal restriction comparable to the sIg of these inclusion positive cells. In this study two morphologically distinct types of inclusion body were observed. The majority of reports describe rod-like crystalline structures which by electron microscopy examination were proven to consist of laminar material with a periodicity of 40 to 60 Å, surrounded by endoplasmic reticulum.^{5 7 9 12} Recently,^{15 28} two reports were published describing a few CLL patients presenting oval shaped inclusions of amorphous material resembling Russell bodies. Guglielmi *et al*²⁸ demonstrated by immuno-electron microscopy that these inclusion bodies consisted of amorphous material surrounded by dilated rough endoplasmic reticulum. Although we did not use their immunological method a similar observation was made in our cases Vdu, Gee and Goo.

As already stated, the immunoglobulin content of both types of inclusion body displayed an identical heavy and light chain restriction to the one present at the surface of the malignant cells.

However, in this context it is important to note that the additional delta chain at the surface of the malignant cells of two patients was not found in the cytoplasmic inclusions. This finding was also noted in the study of Guglielmi *et al*²⁸ and is comparable to findings seen in morbus Waldenström^{29 30} and CLL associated with monoclonal immunoglobulins.^{31 33} In view of our current knowledge on cellular immunoglobulin synthesis³⁴ the former observation indicates that the Ig content of the inclusion bodies is derived from the secretion m-RNA. Indeed, during maturation of the B lymphocyte towards the secreting B cell (ie plasma cell) IgD is lost from the surface.³⁵ This suggests that CLL cells containing this type of inclusion body are more advanced in their maturation level and more closely related to the cells secreting a paraprotein, as is observed in about 5% of all CLL patients.³⁶ It is unclear why the secretory Igs are precipitated in the cytoplasm. This phenomenon could be due to the inability of the Golgi apparatus to add the essential carbohydrate residues for secretion of the Ig.³⁷ This hypothesis is supported by the PAS negativity of the inclusion bodies seen in all our patients. It is intriguing that in most of the patients reported by others and ourselves the inclusion bodies contain lambda light chain. This is in contrast to the predominance of kappa light chain on the cell surface membrane in B-CLL patients.³⁵ Biosynthetic studies^{14 15 38} have shown that monoclonal Ig chains could accumulate and precipitate as crystals due to unusual physicochemical properties, possibly linked to the intracellular environment. It could be hypothesised that lambda light chain possesses such properties.

Finally, the evolution of the percentage of sIg positive and crystal positive lymphocytes of case Cla, during chlorambucil treatment, reflects a different drug sensitivity within the malignant B cell clone (Fig. 6). Only after a certain lapse of time the B cells will accumulate intracellular Ig, which then will precipitate as inclusion bodies. Another possibility could be that the secretory properties of the malignant B cells are affected by chlorambucil. In any case, the final prognosis of patients with CLL and inclusion bodies, does not seem to differ from that of classical CLL. This was proven by the fact that all our 12 CLL patients could be classified from stage I to stage V of the anatomicoclinical classification proposed by Binet *et al*.²⁰ The clinical evolution of these patients was similar to that seen in other CLL patients.

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