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Two Myeloma Globulins IgG1-κ and IgG1-λ, from a single patient (Im)

II. THEIR COMMON CELLULAR ORIGIN AS REVEALED BY IMMUNOFLUORESCENCE STUDIES

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Summary. The patient's (Im) serum contained two myeloma proteins possessing the same heavy chains (γ1) and different light chains (κ or λ).

The presence of identical antigenic determinants in the variable region of the heavy chains of both proteins raises the problem of their cellular origin. The immunofluorescence technique on bone marrow smears was used to identify the cells producing these proteins. Rabbit antisera monospecific to human κ and λ types of light chains, labelled with fluorescein or rhodamine, were applied onto the smears. These studies always revealed the presence of both κ and λ chains in the same plasma cells, thus suggesting that single cells of this patient are capable of synthesizing both IgG1(κ) and IgG1(λ) proteins.

INTRODUCTION

In the preceding paper we have shown that two distinct M components were present in the serum of a myeloma patient (Im), i.e. IgG1(κ) and IgG1(λ) (Oriol, Huerta, Bouvet and Liacopoulos, 1974).

The heavy chains of both proteins were antigenically identical and shared the same individually specific antigenic determinant (*ind*). Light κ and λ chains isolated from each M component possessed different *ind* determinants. The identity of *ind* determinants located in the variable region of the heavy chains of both proteins suggested that these M components could be produced by, or at least originated from, the same ancestor cell.

Previous detailed studies on double myeloma proteins found in the same individuals, showed an identity of some portions of the variable regions of the heavy or light chains. Nisonoff, Wilson, Wang, Fudenberg and Hopper (1971), reported that IgG (κ) and IgM (κ) immunoglobulins from a myeloma patient (Til), had identical light chains and a portion of amino acid sequence of heavy chain variable regions. Yagi and Pressman (1973) showed that IgA (κ) and IgM (κ) isolated from the serum of their patient (SC) shared the same *ind* determinants, thus indicating the presence of some common antigenic regions in the IgA and IgM globulins. The identity in the primary structure of some portions of variable regions in these pairs of M components, suggested in both groups that the cells producing these proteins were derived from a common precursor in spite of the fact that

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each of these M components was produced in different cells (Nisonoff *et al.*, 1971; Silverman, Yagi, Pressman, Ellison and Tormey, 1973).

In some cases of double myeloma proteins it was proved that both M components were produced by the same cells. Costea, Yakulis, Libnoch, Pilz and Heller (1967) found that most of the plasma cells of their patient contained both IgG and IgA globulins; Sanders, Fahey, Finegold, Ein, Reisfeld and Berard (1969) studying the cellular origin of the three M components (IgG, IgA and IgM) identified in one patient (E.E.) found that 42·5 per cent of the examined cells contained both γ and μ heavy chains. More recently, Rudders, Yakulis and Heller (1973) described a case of multiple myeloma (Loy) producing IgG and IgA proteins. These immunoglobulins shared the same L chain (λ type) and the *ind* determinants of V regions of both H chains were identical. Cytological examination showed that 60 per cent of the myeloma cells contained IgG, 30 per cent contained IgA, but 10 per cent contained both immunoglobulins and 100 per cent of cells were labelled by the anti-*ind* serum. A similar 100 per cent labelling of the cells producing either IgG-κ or IgM-κ proteins in the patient Til (Nisonoff *et al.*, 1971) was found when the anti-*ind* determinant of heavy chains were used for staining the patient's cells (Levin, Fudenberg, Hopper, Wilson and Nisonoff, 1971).

In the present study we examined bone marrow smears of our patient (Im) whose serum contained IgG1 (κ) and IgG1 (λ) proteins. Fluorescein or rhodamine labelled monospecific anti-κ or anti-λ sera were used and it was found that both IgG1 (κ) and IgG1 (λ) were invariably produced by the same cells.

MATERIALS AND METHODS

Preparation of the cells

Myeloma cells were harvested by puncture of the patient's hip-bone, washed with Hanks's solution spread on microscope slides and then stored at $-70°$ until examined.

Antisera

Purified human Bence-Jones (B-J) proteins of either κ or λ type were used for the immunization of rabbits. Solutions containing 8 mg/ml of these proteins were mixed in equal volumes with Freund's complete adjuvant and injected intradermally to rabbits. On days 15, 16 and 17 the same proteins precipitated with alum (4 mg) were injected subcutaneously and intravenously.

Sera from these rabbits were taken 5 days after the final injection. The gamma-globulin fraction was prepared by precipitation with ammonium sulphate at 50 per cent saturation. This fraction was then absorbed with various heavy chains and the opposite light chain. Anti-λ serum was also absorbed with the patient's IgG1(κ). Specificity control was carried out in Ouchterlony dishes with both antigens (B-J-κ and B-J-λ). It showed a single line of precipitation with the corresponding antigen.

Conjugation of anti-κ and anti-λ gamma-globulins with isothiocyanate of fluorescein and lissamine-rhodamine

Fluorescein isothiocyanate (FITC) was obtained from BDH (British Drug Houses) and rhodamine lissamine from Calbiochem (Los Angeles, U.S.A.).

The conjugation was performed as follows. The globulin precipitate was lyophilized and then dissolved in 0·5 M carbonate–bicarbonate buffer, pH 9·0 (3·7 g of $NaHCO_3$ and

0·6 g of anhydrous Na_2CO_3 made up to 100 ml with distilled water). Protein concentration was 100 mg of globulin in 6 ml of buffer. Fluorochrome was then added (1·5 mg FITC at 4°) or 30 mg of lissamine–rhodamine. FITC conjugates were stored overnight at +4° with mechanical stirring.

Rhodamine conjugates were stored at 17°. The conjugation mixture was then passed through a column of Sephadex G-25 to separate conjugated and non-conjugated fluorochrome. The yield of these labelling procedures is reported in Table 1. The same procedures were used for labelling rabbit immunoglobulins (RGG), obtained from normal rabbit serum and human serum albumin (HSA crystallized, N.B.C., Cleveland, Ohio, U.S.A.) used for control experiments.

TABLE 1

THE DEGREE OF LABELLING OF ANTI-λ AND ANTI-κ RABBIT GAMMA-GLOBULINS AFTER CONJUGATION WITH EITHER ISOTHIOCYANATE OF FLUORESCEIN OR WITH LISSAMINE–RHODAMINE

Specificity	Fluorochrome	Percentage of labelling	Protein concentration (mg/ml)
Anti-λ	Fluorescein	4×10^{-3}	3·5
	Rhodamine	$4{\cdot}85 \times 10^{-3}$	2·9
Anti-κ	Rhodamine	3×10^{-3}	4
	Fluorescein	$4{\cdot}7 \times 10^{-3}$	2

Cell labelling

Frozen slides were treated with absolute alcohol for cell fixation and when dry, solutions of both labelled gamma-globulins were poured on the slides either simultaneously or sequentially. Sequential application of the two reagents permitted examination of the same cells after each labelling. The reagents were left on the slides for half an hour and then washed thoroughly with saline and distilled water.

Microscope examination of the cells

The microscope employed was a Leitz–Orthoplan with a Ploem vertical illuminator. The light source was an OSRAM H BO 200 lamp. Specifications for the filters are reported in Table 2. Suitable adjustment of these filters made it possible to obtain monochromatic images, depending on the wave length used. The cells were examined at high magnification (×1000) with an oil immersion objective, and photographs were taken with a Fuji colour film, of 100 Aza sensitivity.

RESULTS

SPECIFICITY CONTROL OR REAGENTS

All the smears of the patient's hip-bone marrow cells were examined prior to staining. Some of them showed a spontaneous fluorescence and they were consequently eliminated.

In order to ascertain the specificity of the reagents two control series were performed. In the first series, labelling of the cells was attempted by using fluorochrome-labelled RGG or HSA. No labelling at all of the cells was observed. In the second series, bone marrow cells

TABLE 2

SPECIFICATIONS OF THE FILTERS USED FOR FLUORESCENCE MISCROSCOPY

Filter combination	Exciter filters	Barrier filters	Dichroic mirror	Incorporated barrier filters	Origin of fluorescence
I	—	490	No. 2 T_k 455	No. 2 460	IF*+SR†
II	$BG_{38}BG_{12}$	510	No. 3 T_k 495	No. 3 495	IF
III	$BG_{38}BG_{12}$	520	No. 3 T_k 495	No. 3 495	IF
IV	BG_{38}	610	No. 4 T_k 580	No. 4 580	SR

* IF = fluorescein.
† SR = rhodamine.

from patients possessing a myeloma known to produce either κ or λ light chains were treated simultaneously with both labelled antisera. In each case the cells were exclusively labelled with the corresponding antiserum (Table 3). It was considered that under the conditions of the present experiment, the fixation on the myeloma cells of both antisera conjugated with either of the fluorochromes was satisfactorily specific.

TABLE 3

ISOTYPIC SPECIFICATIONS IN ANTISERA USED FOR IMMUNOFLUORESCENCE TESTED WITH MONOCLONAL MYELOMAS. ABSENCE OF NON-SPECIFIC STAINING OF PATIENT 'Im' MYELOMA CELLS

Myeloma	Staining*	Cellular fluorescein
IgGκ	Anti-κ . SR+Anti-λ . IF	Red
	Anti-κ . SR+B-J κ	Absent
IgGλ	Anti-λ . SR+anti-κ . IF	Red
IgAκ	Anti-κ . SR+anti-λ . IF	Red
IgAγ	Anti-κ . SR+anti-λ . IF	Green
Myeloma 'Im'	HSA-SR+HSA-IF	Absent
	RGG-SR+RGG-IF	Absent

* SR = rhodamine-labelled; IF = fluorescein-labelled. RGG = non-immune rabbit gamma-globulins; HSA = human serum albumin; B-J κ = Bence-Jones chain κ proteins.

LABELLING OF Im MYELOMA CELLS

Exposure of the marrow smears to one antiserum showed the existence of ten to fifty stained cells per slide, all of which were of the plasma series. Their diameter was 7–20 μm and their morphology was usually that of immature plasma cells. A few rare cells had a double nucleus. Cytoplasmic red or green fluorescence was usually granular, but sometimes it was spread throughout the cytoplasm and the nucleus.

When the same smears were exposed to the other serum all plasma cells gave a yellow fluorescence. The same yellow fluorescence was found when the two antisera were poured on the slides simultaneously as a mixture. Nearly all cells were labelled by both anti-κ and anti-λ serum, thus exhibiting a yellow fluorescence (Fig. 1). The use of suitable filters made it possible to examine each fluorescence separately. The results were the same no matter to what fluorochrome, the anti-κ or anti-λ sera were conjugated and no matter what the order of their application onto the slides. In some cells the cytoplasm showed a

FIG. 1

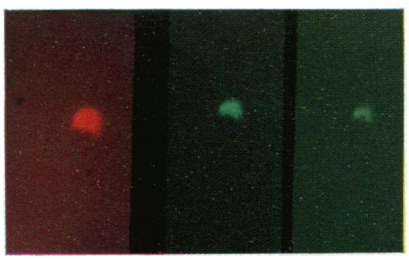
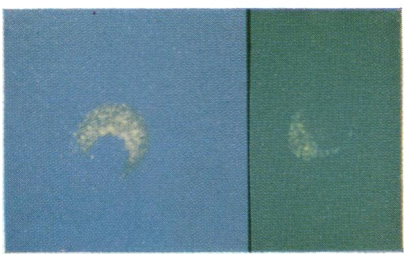


FIG. 2



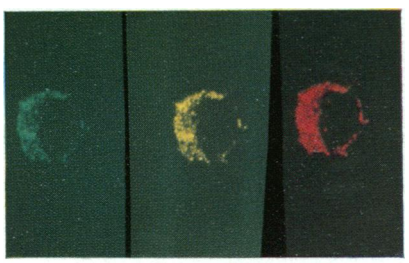


FIG. 3

FIG. 1. One typical plasma cell from patient (Im) stained first with rhodamine (SR) labelled rabbit anti-human λ chain and second with fluorescein (IF) labelled rabbit anti-human κ chain sera. (a) Filter combination IV (Table 2) selective for SR (anti-λ); (b) filter combination III selective for IF (anti-κ); (c) filter combination I allowing observation of both fluorochromes, showing existence of both λ- and κ-specific material throughout the cytoplasm. (Magnification × 1500.)

FIG. 2. Two typical plasma cells from patient (Im) bone marrow stained simultaneously in (a) with anti-κ (SR) + anti-λ (IF) and observed through filter combination I (Table 2) and in (b) anti-λ (SR) + anti-κ (IF) observed through the same filter combination.

FIG. 3. One plasmablast from patient (Im) bone marrow stained first with anti-κ (SR) and second with anti-λ (IF). (a) Filter combination IV (Table 2); (b) filter combination I; (c) filter combination III. As in Fig. 2 material dotted with both κ and λ specificities is patchily distributed throughout the cytoplasm.

diffuse yellow fluorescence (Fig. 1), but in the majority of the cells the fluorescence had a granular appearance (Figs 2 and 3). Subsequent colouration of the cells with May–Grünwald–Giemsa, in order to discriminate whether the later cells belong to the plasma cell series, showed that all the labelled cells were clearly basophilic, although the quality of these second colourations were as usual not adequate for photographs. It was therefore concluded that the cells which did not look as typical plasma cells (Fig. 3) were plasmablasts rather than myeloid cells.

Among the thirty slides examined, only a few monospecific cells were observed. These rare cells containing only one type of the light chains could represent residual normal plasma cells.

DISCUSSION

The assessment of immunoglobulin content of individual cells by immunofluorescent tests requires considerable control stainings in order to ascertain the specificity of the reagents. The rabbit anti-human-κ and λ sera used in the present study were controlled for specificity in several ways: (a) they were produced by immunization of rabbits with purified Bence-Jones urinary proteins either of the κ or of the λ type; (b) tested in Ouchterlony plates, they reacted exclusively with homologous Bence-Jones proteins; (c) smears which exhibit a spontaneous fluorescence were eliminated; (d) some slides were treated with fluorescein-labelled normal RGG or HSA and none was stained (Table 3); (e) bone marrow smears from patients possessing known monoclonal myelomas (IgGκ, IgGλ, IgAλ or IgAκ) were stained with both antisera; in every case the smears were only stained with the corresponding antiserum (Table 3). It can therefore be considered that the constant presence of both κ and λ light chains in the vast majority of cells examined has been proved convincingly. Since immunoglobulin content indicates biosynthetic activity of individual cells, it can be inferred that both κ and λ chains were produced by the same cells. This finding is in agreement with other immunochemical analysis (Oriol *et al.*, 1974), showing that both the myeloma proteins of patient Im shared the same H chains (γ1) possessing identical *ind* determinants.

The simultaneous occurrence of two immunologically different M components is in general rare (1 per cent) (Bouvet, Feingold, Oriol & Liacopoulos, 1975). In those cases where the cellular origin of M components was looked for, it was usually found that they were produced in different cells. In three cases, however, (Costea *et al.*, 1967; Sanders *et al.*, 1969; Rudders *et al.*, 1973) it was found that the same cells simultaneously produced two heavy and one light chains (γ, α, κ; μ, γ, κ and γ, α, λ respectively). On the other hand, detailed immunochemical analysis of V_H globulin portions of double M components showed that significant areas and *ind* determinants were identical in both M components (Prendergast, Gery and Kunkel, 1966; Penn, Kunkel and Gray, 1970; Nisonoff *et al.*, 1971; Yagi and Pressman, 1973; Rudders *et al.*, 1973). Staining myeloma cells with fluorescein conjugated anti-*ind* sera resulted in the labelling of all cells regardless of the class of H chain they produced (Levin *et al.*, 1971; Rudders *et al.*, 1973). It was inferred that in these cases myeloma protein-producing cells originated from the same ancestor cell. Thus, there is increasing evidence that myeloma cells could simultaneously synthesize two different H chains.

This double production should not be attributed to the malignant nature of the myeloma cells. Double producers have been found among normal human cells or cells of immunized

animals (Mellors and Korngold, 1963; Nossal, Szenberg, Ada and Austin, 1965; Burtin and Buffe, 1967; Pernis, Forni and Amante, 1971), although of low incidence (1–2 per cent). These observations have been interpreted as suggesting a genetic switching of C_H genes and their recombination with the same V_H gene.

Simultaneous production of κ and λ light chains has been previously described in twenty cases of double paraproteinaemias (Bouvet *et al.*, 1975). In ten of these cases each type of chain was associated with a different H chain. The cellular localization of these M components were studied in only two cases (Dittmar, Kochwa, Zucker-Franklin and Wasserman, 1968; Rosen, Smith and Bloch, 1967) and showed that M components were produced in different cells. However, no detailed immunochemical analysis has been performed in these cases in order to see whether common structural features between the pairs of immunoglobulins produced in such cases could suggest a common cellular origin. The dual cellular origin of these M components remains the most likely, since two independent pairs of H–L chains were produced simultaneously.

In another ten cases both κ and λ types of L chains were associated with the same H chain (six IgG-κ+IgG-λ, three IgMκ+IgMλ and one IgAκ+IgAλ) (Bouvet *et al.*, 1975). Only in the present case (patient Im), however, has a detailed analysis of IgG1-κ and IgG1-λ M components been undertaken (Oriol *et al.*, 1974).

The identity of the *ind* determinant of γ1 chains of both paraproteins suggested a common genetic origin, and this might indicate that cells belonging to the same clone were synthesizing both proteins.

The finding that *ind* determinants of L chains were different is in agreement with the well known phenomenon that the variable region subgroups are linked to only one of the constant regions (either κ or λ).

The demonstration by immunofluorescence studies that IgG1(κ) and IgG1(λ) molecules were present in the same cells indicates that a given cell at some stage of its differentiation is able to synthesize both κ and λ light chains, although such a cell has not yet been observed in normal or immunized animals.

Simultaneous production of κ and λ L chains by one cell producing one type of H chain raises the problem of the assembling of L and H chains to form complete Ig molecules. Previous studies on synthesis and assembling of Ig suggested that the main pathways for formation of the complete molecule were $H+H \rightarrow H_2+L \rightarrow H_2L+L \rightarrow H_2L_2$ or $H+L \rightarrow HL+HL \rightarrow H_2L_2$ (Baumal and Scharff, 1973). These pathways imply that if the cell produces both κ and λ L chains, hybrid molecules ($H_2L_\kappa L_\lambda$) could be also formed. However, no such molecules were found in detectable quantities in the patient's serum (Oriol *et al.*, 1974). Baumal and Scharff (1973) found that some IgG-producing tumours and cell lines contained covalently bound L_2 and they considered such a pathway possible. They showed that whenever L_2 was present it was an end-product rather than a precursor of H_2L_2. Nevertheless, frequent secretion by cells of light chain dimer (Bence-Jones proteins) which are non-covalently bound (κ_2 or λ_2 dimers) suggest that even when κ and λ chains are formed by the same cell they may first be linked to form homologous dimers, then assembled with H chains, and finally secreted as complete Ig molecules.

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