Immunohistologic Localization of Gamma-1-Macroglobulins, Beta-2A-Myeloma Proteins, 6.6 S Gamma-Myeloma Proteins and Bence Jones Proteins

By Alan Solomon, John L. Fahey and Richard A. Malmgren

It is now generally accepted that at least four classes of related proteins comprise the gamma globulin group—that is, the 6.6 S γ-globulins, β2A-globulins, γ1-macroglobulins and Bence Jones proteins. Many studies indicate that gamma globulin formation occurs in plasma cells and lymphoid cells but the features of the cells in which each of these distinctive classes of globulins are formed are not clear.

Morphologic differences in the cells forming these gamma globulins have been inferred from observations of the predominant cell form seen in malignant disease. For example, γ1-macroglobulinemia is frequently associated with proliferation of lymphoid cells or lymphoid-plasma cells. A variety of plasma cell forms, including those with small, medium or large nuclei, with scanty to abundant cytoplasm, and with other varying morphologic features, have been described in multiple myeloma. Some investigators have found that the electrophoretic mobility of myeloma proteins relates to plasma cell morphology and that large cells with abundant cytoplasm are more frequently associated with myeloma proteins of gamma than of beta globulin mobility.

Olhagen et al. and Le-Xuan-Chat found that Bence Jones proteinuria was frequently associated with immature plasma cell proliferation and, recently, Paraskevas et al. have suggested that β2A-myeloma proteins are associated with large cells of a distinctive cytoplasmic structure and with small pyknotic nuclei. Some investigators, however, have found no correlation at all between the protein characteristics and the cellular features of multiple myeloma. In addition, it was not possible to determine whether all or only some of the malignant cells were forming the anomalous proteins.

Specific cellular localization of gamma globulins became possible with the introduction of the immunofluorescent technic by Coons and Kaplan. Antibody globulin was identified by White and by Coons et al. in plasma cells and lymphoid cells in the lymph node. Ortega and Mellors identified gamma globulin in similar cell forms. In studies with malignant plasma cell disease, Vasquez observed gamma globulin fluorescence in plasma cells of patients with multiple myeloma. Specific macroglobulin fluorescence in
### Table 1.—Characteristics of the Anomalous Proteins and Bone Marrow Morphology in Patients Having Immunohistochemical Studies

<table>
<thead>
<tr>
<th>Patient and Class of Anomalous Globulin Type</th>
<th>Myeloma Protein or Macroglobulin Type</th>
<th>Quantity Mobility (Gm. %)</th>
<th>Excretion (Gm./24 hrs.)</th>
<th>Therapy in Previous Month</th>
<th>Bone Marrow Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. γ-Myeloma Protein</td>
<td>I</td>
<td>L.M. 8.3 -6</td>
<td>none</td>
<td>12 % plasma cells with eccentric nuclei, abundant, foamy cytoplasm and a paranuclear clear zone, some cells with intranuclear inclusions.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>N.S. 7.5 -16</td>
<td>none</td>
<td>30 % plasma cells with considerable anisocytosis with a preponderance of very large cells, absent paranuclear clear zone, immature nuclear chromatin pattern.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>E.S. 9.3 -20</td>
<td>none</td>
<td>70 % large abnormal plasma cells with immature chromatin pattern, abundant cytoplasm, absent paranuclear clear zone.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>T.N. 2.0 +2 6</td>
<td>II prednisone</td>
<td>Plasma cells varying greatly in size, eccentric nuclei, abundant foamy cytoplasm, binucleate and trinucleate forms common.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>O.T. 5.0 -21 1</td>
<td>I none</td>
<td>Plasma cells with moderate amount of foamy cytoplasm, no paranuclear clear zone.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>L.E. 4.2 -24 4</td>
<td>II none</td>
<td>Areas of small plasma cells with scanty cytoplasm, some resembling atypical lymphocytes.</td>
<td></td>
</tr>
<tr>
<td>II. β,-Myeloma Protein</td>
<td>II</td>
<td>G.F. 2.8 -6</td>
<td>none</td>
<td>35 % plasma cells, many large and binucleate forms.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>J.G. 3.5 +10 1</td>
<td>I none</td>
<td>15 % plasma cells of uniform morphology, medium to large in size with abundant foamy cytoplasm and eccentric nucleus with reticular chromatin pattern.</td>
<td></td>
</tr>
<tr>
<td>III. Bence Jones Protein</td>
<td>II</td>
<td>Z.O. +15 27</td>
<td>II none</td>
<td>10 % plasma cells containing eccentric nuclei with clumped chromatin, moderate amount of cytoplasm with a paranuclear clear zone.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>E.B. +15 7.5</td>
<td>I prednisone</td>
<td>Majority of immature plasma cells with abundant cytoplasm which frequently contains eosinophilic granules.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>S.E. +10 7</td>
<td>6-MP prednisone</td>
<td>Malignant cells resembling reticulum cells with ragged, scanty cytoplasm and powdery-to-reticular nuclear chromatin.</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1.—(Continued)

<table>
<thead>
<tr>
<th>Patient and Class of Anomalous Globulin</th>
<th>Myeloma Protein or Macroglobulin Type</th>
<th>Quantity</th>
<th>Excretion (Gm./24 hrs.)</th>
<th>Therapy in Previous Month</th>
<th>Bone Marrow Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quan- (Gm., %)</td>
<td>Mobility Excretion (mm. from origin)</td>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.P.</td>
<td>+4</td>
<td>4</td>
<td>I</td>
<td>none</td>
<td>75 % young plasmacytes with eccentric nuclei, reticular dense chromatin, foamy cytoplasm.</td>
</tr>
<tr>
<td>D.R.</td>
<td>+2</td>
<td>30</td>
<td>II</td>
<td>none</td>
<td>Medium sized malignant mononuclear cells with central nuclei, clumped chromatin, scanty cytoplasm, some cells more typical plasma cells.</td>
</tr>
<tr>
<td>IV. γ, Macroglobulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.C.</td>
<td>I 6.0</td>
<td>+10</td>
<td>chlorambucil</td>
<td>25 % plasma cells with marked anisocytosis, some with intranuclear inclusions, 35 % small and medium sized lymphocytes.</td>
<td></td>
</tr>
<tr>
<td>M.G.</td>
<td>I 2.6</td>
<td>-3</td>
<td>chlorambucil</td>
<td>15 % mature lymphocytes, 5 % plasma cells and 8 % lymphoid plasma cells, eccentric nuclei with reticular chromatin pattern.</td>
<td></td>
</tr>
<tr>
<td>J.B.</td>
<td>I 3.3</td>
<td>-10</td>
<td>prednisone</td>
<td>15 % small and medium sized lymphoid forms with plasmacytic features.</td>
<td></td>
</tr>
<tr>
<td>J.W.</td>
<td>I 4.5</td>
<td>-13</td>
<td>chlorambucil</td>
<td>20 % mature appearing plasmacytic cells with small amount of cytoplasm.</td>
<td></td>
</tr>
<tr>
<td>L.H.</td>
<td>I 1.0</td>
<td>-15</td>
<td>none</td>
<td>15 % lymphocytes of medium size, occasional plasma cells.</td>
<td></td>
</tr>
<tr>
<td>W.F.</td>
<td>I 5.0</td>
<td>-20</td>
<td>none</td>
<td>Lymphocytic cells of various sizes, many with scanty cytoplasm; clumped nuclear chromatin.</td>
<td></td>
</tr>
<tr>
<td>F.R.</td>
<td>II 1.2</td>
<td>+15</td>
<td>4</td>
<td>II</td>
<td>none</td>
</tr>
<tr>
<td>M.T.</td>
<td>I 6.5</td>
<td>-5</td>
<td>0.3</td>
<td>prednisone</td>
<td>50 % relatively young plasmacytes with large nuclei, some lymphoid in appearance, and 10 % lymphocytes.</td>
</tr>
</tbody>
</table>

*Mobility of the urinary Bence Jones protein is given in this section.

Lymphoeytoid plasma cells have been reported by Curtain and O’Dea, Dutcher and Fahey, Curtain, Burtin, Kritzman et al., and Zucker-Franklin et al. Some of these reports were limited to studies of only a few patients, and antisera specific for each globulin may not have been employed. Furthermore, these reports concentrated on one globulin type, and morphologic material associated with all four classes of gamma globulin was not compared.

In the present work, the specific localization of the four classes of gamma globulin (6.6 S γ, β₁₂, γ₁-macroglobulins and Bence Jones proteins) were studied concurrently. Immunohistochemically specific antisera and immunofluorescent techniques were used to locate cells containing each type of protein.
Morphologic material from 21 patients known to be forming large amounts of specific protein—that is, patients with multiple myeloma or macroglobulinemia—was examined and the fluorescent antibody results were directly related to the morphology of similar cells seen by Giemsa-staining.

Materials and Methods

Patients Studied

Of the 21 patients included in this study, 13 had multiple myeloma and eight had macroglobulinemia. All patients were under our observation, and the diagnoses were established by clinical and morphologic criteria and by appropriate serum and urine protein analyses (table 1).

Serum from each patient was examined by paper electrophoresis in order to determine the mobility and quantity of the anomalous protein. The type of anomalous protein in the serum and urine was determined by immunoelectrophoresis in which we used a polyvalent rabbit antiserum prepared in our laboratory. This antiserum was shown to react with 6.6 S γ-globulins, β2γ-globulins and γ1-macroglobulins in normal serum and to react with serum γ-myeloma proteins, β2α-myeloma proteins, macroglobulimemic γ1-macroglobulins and Bence Jones proteins in multiple myeloma and macroglobulinemia.28 We also employed rabbit antisera specific for human 6.6 S γ-globulin, β2γ-globulin or γ1-macroglobulin to confirm the anomalous protein classification.28 Ultracentrifugation was performed on all macroglobulimemic sera, β2α-myeloma sera and on Bence Jones proteins.29 In each case the anomalous protein was isolated and characterized by physicochemical and immunochemical technics. Of the 13 patients with multiple myeloma, three had 6.6 S γ-myeloma proteins, three had 6.6 S γ-myeloma protein and Bence Jones proteinuria, one had β1α-myeloma protein, one had β2α-myeloma protein and Bence Jones proteinuria, and five had Bence Jones proteinuria without demonstrable γ or β2α-myeloma serum protein. Eight patients had macroglobulinemia, with serum γ1-macroglobulin levels ranging from 1 to 7 Gm. per cent. Two of these eight patients had Bence Jones proteinuria in addition to macroglobulinemia.

Source and Preparation of Morphologic Material

Bone marrow aspirates were obtained from all patients. A portion of the aspirate was smeared on cover slips and treated with the conventional Wright-Giemsa stain. The remainder was used for immunofluorescent studies. This portion was placed in a 15 ml. centrifuge tube containing heparin and 10 ml. of a 5 per cent human albumin solution in buffered saline pH 7.4. After centrifugation at 300 g for 10 minutes the sediment and any floating spicules were saved and the supernatant, containing contaminating serum proteins, was discarded. The washing procedure was repeated twice. The marrow material was then smeared on cover slips, air-dried and fixed in acetone at room temperature for 15 minutes. A sufficient number of cover slips were prepared to permit testing with a variety of antisera. The cover slip preparations were then placed in separate humidified Petri dishes at room temperature and flooded with specific rabbit antisera against human globulins. After 1 hour the rabbit antisera were washed off and the cover slips were rinsed three times with phosphate buffered saline pH 7.4. To determine where specific rabbit anti-human globulin antisera had been fixed to the cells, fluoresceinated sheep anti-rabbit globulin antiserum (previously absorbed with acetone-insoluble mouse liver powder) was applied at room temperature for 1 hour.4 The cover slips were again washed three times with buffered saline and mounted in 25 per cent glycerol. Control slips were also prepared by using the fluoresceinated sheep antiserum directly, without first applying the rabbit anti-human globulin antisera.

Lymph node biopsies as well as marrow aspirates were obtained from two of the patients with macroglobulinemia. One portion of the node was fixed in formalin for routine hematoxylin-eosin stain. The other portion of the node was immediately frozen at 70°C,
with a dry ice-isopropyl alcohol mixture. Cryostat sections were placed on cover slips, air-dried and then fixed in acetone for 15 minutes. The application of antisera, etc. was similar to the immunofluorescent procedure of examining the bone marrow preparations.

The nuclear size and cell size of the abnormal cells were measured in the Giesma-stained preparations by means of a eyepiece micrometer. Approximately 50 such cells in each patient were measured.

Fluorescent microscopy was carried out using a Leitz microscope and an ultraviolet light source of 390 to 440 μμ wave length. The filters used were a BG-12 transmitting filter and an OG-1 absorbing filter. Photographs of the immunofluorescent preparations were taken with Kodak Tri-X 35 mm. film with an exposure time of 1 minute.

**Antisera Reacting with Gamma Globulin Components**

Antisera were produced in rabbits by immunizing them with purified human gamma globulin components and Freud’s adjuvant. Whole gamma globulin (prepared by zone [block] electrophoresis) of normal serum, normal 6.6 S γ-globulin (prepared by DEAE-cellulose chromatography), normal and macroglobulinemic 18 S γ1-macroglobulins, and purified β2A-like myeloma proteins were used as antigens. Antiserum specific for 6.6 S γ-globulin was prepared by absorbing anti-6.6 S γ-globulin antiserum with purified β2A-like myeloma proteins and macroglobulinemic γ1-macroglobulins. Antiserum specific for β2A-like globulins was prepared by absorbing anti-β2A globulin antiserum with normal 6.6 S γ-globulins and macroglobulinemic γ1-macroglobulins. Specific anti-18S γ1-macroglobulin antiserum was prepared by absorbing anti-macroglobulin antiserum with 6.6 S γ-globulins and β2A-like globulins. Specificity of these antisera was checked by immunoelectrophoresis against normal human serum and sera containing γ-myeloma proteins, β2A-like myeloma proteins or macroglobulinemic macroglobulins, as well as by Ouchterlony double diffusion tests with purified individual gamma globulin components. Additional details of the preparation and reactivity of these antisera are presented elsewhere.

Both polyvalent and specific types of antisera were used in the present study. A polyvalent antiserum (R33) reacting strongly with all types of gamma globulin (6.6 S γ, β2A, γ1-macroglobulins, and Bence Jones proteins) was used to identify cells containing any type of gamma globulins. Specific antisera reacting with 6.6 S γ-globulins (R33A), with β2A-like globulins (R43A, R56A) or with γ1-macroglobulins (R36A, R11A) were used to identify cells containing specific gamma globulin components.

All of the Bence Jones proteins reacted with the polyvalent anti-γ-globulin antiserum, R33. Bence Jones proteins were identified by comparing the results obtained in cellular preparations treated with R33 antiserum and with R33 antiserum absorbed with the patient’s own Bence Jones proteins. The absorbed antiserum no longer reacted on agar diffusion tests with the Bence Jones protein. Control tests showed that only the antigenic determinants of Bence Jones protein had been removed by absorption and that the antiserum still contained many antibodies against other antigenic determinants on 6.6 S γ-globulins. The Bence Jones protein absorbed antiserum still reacted strongly with γ-globulins in agar diffusion tests. Although the cellular localization of Bence Jones proteins was indirect, the differences between the positive and negative reacting antisera were readily evident in the cellular material from patients with Bence Jones protein as the anomalous protein. Two types of Bence Jones proteins, differing in antigenic composition and designated as I and II, were identified. The cellular locations of both types of proteins were made by the technic outlined above.

**Results**

**Cells Containing Gamma Globulin**

The polyvalent anti-γ-globulin antiserum, R33, reacting with all four classes of gamma globulins (6.6 S γ, β2A, γ1-macroglobulins and Bence Jones proteins), was used to identify cells containing gamma globulin components. A variety
of cellular forms showed bright cytoplasmic fluorescence, which indicated the presence of gamma globulins. These cells included: a) plasma cells characterized by a small eccentrically located nucleus and plentiful cytoplasm; b) cells with a larger and more centrally placed nucleus and with abundant cytoplasm; c) large cells with a large central nucleus and scanty cytoplasm; and d) lymphocytoid-plasma cells with nuclear and cytoplasmic features intermediate between lymphocytes and plasma cells (figs. 1–5). Giemsa-stained preparations of these cells indicated considerable variation in their morphologic detail. Specific antisera were used to identify cells associated with each of the several classes of gamma globulins.

6.6 S γ-Myeloma Protein (Three Patients)

Bright cytoplasmic fluorescence identified 6.6 S γ-myeloma protein in the
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cytoplasm of plasma cells as seen in figure 1. The relative amount of cytoplasm and the size of the nucleus varied from patient to patient. Many of the cells had abundant cytoplasm and a small eccentrically-located nucleus (fig. 1a). Plasma cells in the Giemsa-stained preparations contained small, eccentrically-located nuclei which had an immature chromatin pattern and one or more large nucleoli. The cytoplasm was abundant, deeply basophilic and rarely contained a paranuclear clear zone. Occasionally, some plasma cells in all three patients were extremely large with a diameter of 25 μ. The cytoplasm of these cells fluoresced as extensively as in the small plasma cells. Binucleated plasma cells were commonly seen and also showed cytoplasmic fluorescence. Russell bodies were not observed in the plasma cells treated with the Giemsa stain or fluorescent technics.

Evidence that these cells contained only 6.6 S γ-type globulin was obtained by using several different specific antisera. Antisera specific for β2μ-globulin or γ1-macroglobulin gave negative reactions, in contrast to the bright cytoplasmic fluorescence observed when specific anti-γ-globulin antisera was employed.

Fig. 2.—Immunohistologic observations in a patient with β2μ-myeloma proteins. a) Morphologic variety of Giemsa-stained plasma cells (patient G. F. x 1000); b) cytoplasmic fluorescence with specific anti-β2μ-globulin antiserum in a large plasma cell with a large nucleus (patient G. F. x 1200); c) small plasma cell with specific cytoplasmic fluorescence (patient G. F. x 1200).
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\(\beta_{2\lambda}\)-Myeloma Protein (Two Patients)
The intracellular presence of \(\beta_{2\lambda}\)-myeloma protein in plasma cells was revealed by bright cytoplasmic fluorescence with the use of specific anti-\(\beta_{2\lambda}\)-globulin antiserum (fig. 2). Variation in nuclear size and quantity of cytoplasm also was noted among the fluorescent cells. However, the fluorescent cells generally contained abundant cytoplasm. Plasma cells with similar general morphologic characteristics were seen on the Giemsa-stained marrow preparations from these two patients. The plasma cells varied in size as well as in their nuclear and cytoplasmic features. The nuclei of some plasma cells appeared relatively mature, and in other cells the nuclei had a reticulated chromatin pattern and contained a single, large nucleolus. The abundant basophilic cytoplasm of many plasma cells was multivacuolated. In one patient some plasma cells had an intensely eosinophilic area in the peripheral cytoplasm. Concentration of fluorescence in the periphery of the cytoplasm was also noted in occasional plasmacytic cells in this patient (fig. 2c). The nucleus of some plasma cells was eccentrically-located, and in other cells was larger and more centrally located. However, in both of these patients and in Giemsa-stained marrow preparations from six other patients with \(\beta_{2\lambda}\)-myeloma proteins, there was no clear-cut distinction between the morphologic characteristics of the plasma cells and the plasma cells of patients with \(\gamma\)-myeloma proteins. This morphologic range of cell types was also readily apparent in the immunofluorescent preparations where a variety of plasma cells were stained with the specific antiserum.

The positive intracellular identification of \(\beta_{2\lambda}\)-type protein was confirmed by the fact that fluorescence occurred only with the specific anti-\(\beta_{2\lambda}\)-globulin antiserum and by the fact that no fluorescent reaction occurred with the specific anti-6.6 S \(\gamma\) or anti-\(\gamma_1\)-macroglobulin antisera.

\(\gamma_1\)-Macroglobulins (Six Patients)
Intracellular \(\gamma_1\)-macroglobulins were identified by specific immunofluorescence (fig. 3). Correlation of the positive fluorescent cells with the Giemsa-stained marrow preparations revealed that \(\gamma_1\)-macroglobulins were present in typical plasma cells, in medium to large size lymphocytes, and in cells having the morphologic features of both cell types. The latter cells, described as lymphocytoid plasma cells, had a large centrally located nucleus with a finely reticulated or smooth chromatin pattern (fig. 3a).

\(\gamma_1\)-macroglobulin, as evidenced by specific fluorescence, was evenly distributed throughout the cytoplasm of lymphocytoid plasma cells. The fluorescence appeared more intense in these cells than in the smaller, more typical plasma cells. In the latter cells the small nuclei were eccentrically located and contained dense chromatin patterns. The cytoplasm was vacuolated and frequently contained a paranuclear clear zone. In some plasma cells the fluorescence was located at the periphery of the cells. In an occasional cell a prominent paranuclear clear zone lightly fluoresced.

Antiserum prepared against a macroglobulinemic \(\gamma_1\)-macroglobulin as well as against normal \(\gamma_1\)-macroglobulin gave positive fluorescent reactions. Specif-
immmunohistologic localization was demonstrated by the lack of fluorescence with specific anti-γ and anti-β<sub>2</sub>-globulin antisera.

In one patient, M. G., 6.6 S γ-globulin was demonstrated with the use of specific antisera in the cytoplasm of small plasma cells. The fluorescent reaction, although weak, was considered positive and was not seen to occur in the lymphocytoid cells. Neither type cell was seen to react with the specific anti-β<sub>2</sub>-globulin antiserum. Quantitative measurements revealed that the 6.6 S γ-globulins were considerably higher (1.2 Gm. per cent) in this patient than in the other macroglobulinemic patients.

Immunofluorescent studies were also carried out on lymph node preparations from two of these patients (L. H. and W. F.). With specific antisera, γ<sub>1</sub>-macroglobulin was identified in the cytoplasm of lymphoid cells. Examination of the hematoxylin-eosin stained node of patient W. F. showed replacement of the lymph node architecture by small lymphocytic cells with large central nuclei and scanty cytoplasm as well as by larger forms which resembled lymphocytes but which had nuclear features of plasma cells. Both types of cells were seen in his bone marrow. The immunofluorescent preparations from both the lymph node and bone marrow showed the presence of γ<sub>1</sub>-macroglobulin in the lymphoid-plasma cells. γ<sub>1</sub>-macroglobulin was not identified in the small lymphocytes. 6.6 S γ-Globulin was identified in occasional cells with more typical morphologic features of plasma cells. β<sub>2</sub>A-globulin was not identified in the material examined. Similar observations were made in the lymph node and bone marrow preparations from patient L. H.

**Bence Jones Proteins (Five Patients)**

In the bone marrow of patients with Bence Jones proteinuria, the polyvalent anti-gamma globulin antiserum, R33, revealed cytoplasmic fluorescence in a variety of cells ranging from plasmacytic to lymphocytic in their gross morphology (fig. 4). When this antiserum was absorbed with the individual patient's Bence Jones protein, no fluorescence occurred, indicating that the positive reaction with unabsorbed antiserum was due to the binding of the antibodies to Bence Jones protein in the cytoplasm. This Bence Jones protein absorbed antiserum could still react with 6.6 S γ-globulin when tested by agar-diffusion or immunoelectrophoresis, indicating that the absorbed antiserum had lost only antibodies directed against the Bence Jones protein. Further proof that Bence Jones protein absorbed antiserum could react with gamma globulins lies in the fact that positive immunofluorescence was obtained in plasma cells from patients with γ-myeloma proteins. The positive results obtained with whole R33 antiserum and the negative results obtained with antisera absorbed with the autologous Bence Jones protein led to the indirect intracellular demonstration of Bence Jones proteins in all five patients.

Examination of the Giemsa-stained bone marrow preparations from the patients in this group clearly indicated a morphologic variety of plasma cells both in the individual patient and within the group. In two individuals (H. P. and E. B.) the malignant cells were 11 to 15 μ in diameter with a nucleus
Fig. 3.—Immunohistologic observations in patients with γ1-macroglobulins. a) Giemsa-stained preparation—a large lymphocytoplasmic fluorescence with specific anti-γ1-macroglobulin antiserum in a cell with a centrally located nucleus (patient W. F.); c) specific fluorescence in macroglobulin-containing plasma cells (patient M. T.); d) Giemsa-stained preparation—a morphologic variety of plasma cells (patient M. T.); e) Giemsa-stained and f) g) fluorescent preparations of cells containing γ1-macroglobulin (patient M. G.).
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of 8 to 10 \( \mu \) in diameter. The majority of tumor cells in these two patients were lymphoid in appearance with nuclei that occupied the center of the cell (figs. 4a, g). The nuclei had coarse, clumped chromatin patterns and occasionally contained a large nucleolus. More typical plasma cells with eccentrically located nuclei were also present in the marrow of these two patients. The cytoplasm of the plasma cells was vacuolated with a clear paranuclear zone. Multinucleated forms were also seen. Bence Jones proteins, indicated by immunofluorescence, were present in the cytoplasm of cells similar to the lymphoid cells as well as in the more typical plasma cells seen in the Giemsa-stained preparations. The fluorescence was evenly distributed throughout the cytoplasm of these cell types, although in some of the cells with an eccentrically-located nucleus an area around the nucleus did not fluoresce.

A third patient, D. R., excreted 20 to 30 Gm. of Bence Jones protein in his urine daily. He had numerous osteolytic lesions and pathologic fractures. Examination of bone marrow sections and smears showed complete replacement of normal marrow elements by lymphoid cells (fig. 4b). These cells had centrally located nuclei which occupied almost the entire cell. The nuclei showed clumping of chromatin and, occasionally, a distinct nucleolus. Some areas of the smear contained cells that were larger and more typical of plasma cells. Immunofluorescent studies showed Bence Jones proteins in both the lymphoid cells (fig. 4c) and in the plasma cells. Fluorescence was evenly distributed throughout the thin rim of cytoplasm in the lymphoid cells and yet varied in intensity from cell to cell.

The fourth patient, Z. O., excreted 30 Gm. of Bence Jones proteins in her urine daily and had extensive osteolytic lesions with pathologic fractures. Her bone marrow differed from the preceding patient in that the predominant cell type seen in the Giemsa-stained preparation was clearly plasmacytic with a small eccentrically located nucleus and abundant cytoplasm giving a positive immunofluorescent reaction for Bence Jones proteins.

The fifth patient, S. E., with typical clinical features of multiple myeloma, including extensive osteolytic lesions with pathologic fractures and hypercalcemia, had a bone marrow infiltrated by large immature cells in addition to smaller, more typical plasma cells. The nuclear chromatin of the immature cells was arranged in a fine reticular pattern. These cells were thought to be reticulum cells. Immunofluorescent studies showed Bence Jones proteins to be present in the reticulum cells as well as in the plasma cells.

Two distinct types of Bence Jones protein were found in these patients. Patients H. P. and E. B. had type I Bence Jones proteins and D. R., Z. O. and S. E. had type II Bence Jones proteins. Antisera absorbed with type I Bence Jones proteins did not react with any malignant cells in patients with type I Bence Jones proteins, but could still react with cells of patients producing type II Bence Jones proteins. Antisera absorbed with type II Bence Jones proteins reacted only with type I Bence Jones proteins. The morphologic characteristics of the plasma cells associated with type I Bence Jones proteins could not be shown to differ from those of type II Bence Jones proteins.
Fig. 4 — Immunohistologic observations in patients with two types of Bence Jones proteins. a) Small cells with large nuclei and scanty cytoplasm. Bence Jones protein (Type I) indicated by cytoplasmic fluorescence. b) Small cells with prominent nucleoli. Bence Jones protein (Type II) indicated by cytoplasmic fluorescence. e) Plasma cell with similar morphology—cytoplasmic fluorescence. f) Germin-stained preparation of cell with evident nuclear pyroninophilia. g) Immunofluorescent preparation of cell with evident nuclear pyroninophilia. h) Immunofluorescent preparation of cell with evident nuclear pyroninophilia.

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With the exception of one patient, H. P., no cellular fluorescence occurred with the specific anti-6.6 S γ, β₂₅ or macroglobulin antisera. All five patients had decreased serum levels of these globulins. In H. P., 6.6 S γ-globulin was identified in occasional cells which had similar gross morphologic characteristics to the cells that contained Bence Jones proteins.

6.6 S γ-Myeloma Protein and Bence Jones Proteins (Two Patients)

Morphologic material was evaluated separately from patients having Bence Jones proteinuria in addition to anomalous serum proteins. Particular attention was directed to the question as to whether Bence Jones proteins were formed in the same cells as were the larger globulins, or whether they were formed in different cells.

The Giemsa-stained bone marrow preparations from two patients with γ-myeloma protein and Bence Jones proteins showed infiltration of malignant cells containing small eccentrically located nuclei with moderate amounts of cytoplasm (fig. 5). Other malignant cells had centrally located nuclei which resembled lymphocytes. Approximately 20 per cent of these malignant cells had large immature nuclei that occupied almost the entire cell.

In both types of cells, gamma globulins were identified with the polyvalent anti-gamma globulin antiserum, R33. Positive fluorescence also was obtained with antiserum, R33, absorbed with the patient's Bence Jones proteins and indicated the presence of γ-myeloma protein in the cells. This absorbed antiserum no longer reacted with the Bence Jones proteins but continued to react with the γ-myeloma protein. The presence of γ-myeloma protein was confirmed by the positive fluorescent results obtained with specific anti-6.6 S γ-globulin antiserum (fig. 5c). This antiserum did not react with the isolated Bence Jones protein, nor with β₂₅-globulin or γ₁-macroglobulin. All the malignant cells showed fluorescence with the specific anti-γ₁-globulin antiserum, indicating that all of the malignant cells were involved in myeloma protein formation.

The site of Bence Jones protein formation could not be demonstrated directly by the immunofluorescent technics because the antigenic determinants on these Bence Jones proteins were shared with the γ-myeloma proteins, preventing the preparation of specific antiserum to Bence Jones proteins. However, the cellular distribution of positive fluorescence obtained with the polyvalent antiserum, R33 (which reacted with Bence Jones proteins and γ-myeloma proteins), and the positive fluorescence obtained with specific antiserum (which reacted only with γ-myeloma proteins) were the same. No cellular fluorescence was noted with the use of specific anti-β₂₅ or anti-γ₁-macroglobulin antisera. These results indicate that Bence Jones proteins are present in the same cells as the myeloma protein.

γ₁-Macroglobulins and Bence Jones Proteins (Two Patients)

Cellular morphology and immunofluorescent staining characteristics in this group were similar to those described above in six patients with macroglobulinemia alone. γ₁-macroglobulins were identified in a variety of plasmocytic and lymphoid cells by immunofluorescent staining using specific anti-γ₁-
Fig. 5.—Immunohistologic observations in a patient with \(\gamma\)-myeloma protein and Bence Jones protein. a) Giemsa-stained preparation—small plasma cells with scanty cytoplasm (patient L. E. x 1000); b) similar type cells containing \(\gamma\)-myeloma protein and Bence Jones protein indicated by fluorescence with polyvalent anti-\(\gamma\)-globulin antiserum, no fluorescence with specific antisera to \(\beta_2\lambda\) or \(\gamma_1\)-macroglobulins; c) \(\gamma\)-myeloma protein indicated by fluorescence with Bence Jones protein-absorbed polyvalent anti-\(\gamma\)-globulin antiserum (patient L. E. x 1200).

macroglobulin antisera (R36A and R11A) which did not react immunochemically with the patient's Bence Jones proteins. Direct demonstration of Bence Jones proteins was not feasible because the Bence Jones proteins shared antigenic determinants with the macroglobulins. Thus, antisera reacting specifically with Bence Jones protein could not be obtained. Tests with anti-\(\gamma\)-globulin antiserum absorbed with Bence Jones proteins showed reduced fluorescence. Blocking tests also were compatible with the presence of Bence Jones proteins in the plasmocytic and lymphoid cells. In the blocking tests marrow material was first treated with specific anti-\(\gamma_1\)-macroglobulin antiserum, R36A, to combine with (block) the \(\gamma_1\)-macroglobulin specific antigenic determinants on the macroglobulin molecules. This was followed by application of fluorescein-labeled anti-gamma globulin antiserum (which reacted with Bence Jones proteins). This antiserum gave similar positive cellu-
lar fluorescence in blocked and unblocked preparations. But since this antiserum reacted with the common antigenic determinants on \( \gamma_1 \)-macroglobulin molecules as well as with the antigenic determinants on Bence Jones proteins, it was not certain that the positive fluorescence was due solely to the presence of Bence Jones proteins in the cells. When this antiserum was absorbed with Bence Jones proteins it did not react with the cellular material blocked with R36A.

No fluorescence was seen with specific anti-\( \beta_2 \)-globulin antiserum and only a few, more typical appearing plasma cells reacted with specific anti-6.6 S \( \gamma \)-globulin antiserum. Although these studies do not constitute proof of the existence of Bence Jones proteins and macroglobulin in the same cells, the findings are compatible with this interpretation.

**DISCUSSION**

The cellular sites of gamma globulin synthesis have been indirectly identified. Biosynthesis of gamma globulin components has been demonstrated in appropriate normal or malignant tissues by means of isotopic biochemical technics. Biochemical technics do not demonstrate synthesis in individual cells. Immunohistochemical technics, although showing the presence of a specific protein in single cells, do not demonstrate the cellular synthesis of that protein. However, the evidence that tissues synthesizing gamma globulins contain fluorescent positive cells (when anti-gamma globulin reagents are used in the immunofluorescent test) supports the view that the immunohistochemically positive cells are the sites of gamma globulin synthesis. A variety of morphologic forms of plasma cells and lymphoid cells have been found to contain gamma globulins and antibodies.

Gamma macroglobulins have been identified in a variety of circumstances. Macroglobulin rheumatoid factors were identified by Mellors et al. in immature, mature and Russell body-containing plasma cells in inflammatory exudates near the joint. These factors were also found in similar plasma cells as well as in lymphoid, germinal center cells of hyperplastic lymph nodes from patients with active rheumatoid arthritis. Cruchaud et al. identified \( \gamma_1 \)-macroglobulin in transitional cell forms in the spleen of a patient with dysgammaglobulinemia and in splenic tissue from normal subjects. Curtain and O'Dea noted macroglobulins in mature and immature plasma cells of the bone marrow in a patient with Mikulicz's syndrome. Macroglobulinemic macroglobulins were identified in plasmablasts but not in mature plasma cells and in lymphocytoidal plasma cells in the bone marrow of individual cases of Waldenström's macroglobulinemia. Kritzman et al. identified macroglobulins in lymphocytoidal cells and in plasma cells present in the bone marrow, lymph node, spleen and peripheral blood of a patient with macroglobulinemia. Burtin described macroglobulins in lymphoid cells but not in the typical plasma cells in lymph nodes of six patients with Waldenström's macroglobulinemia. Zucker-Franklin and associates studied lymph nodes, bone marrow and circulating leukocytes in three patients with macroglobulinemia. Cytoplasm of medium and large-sized lymphocytes and lymphoid
reticulum” cells fixed the immunofluorescent stain, but small lymphocytes did not.

In the present study, $\gamma_1$-macroglobulins were identified in a variety of lymphoid and plasma cells of mature and immature forms. None was seen in small lymphocytes. Lymphocytoid plasma cells were frequently encountered in patients with macroglobulinemia, more commonly than in patients with malignancies associated with other types of anomalous proteins. This lymphocytoid cell type, however, was not predominant in all cases of macroglobulinemia. In patient M. T., for example, most of the malignant cells were identified as belonging to the plasma cell series.

Antibody globulin (presumably 6.6 S $\gamma$-globulin) has been identified in plasma cells of hyperimmunized animals. Ortega and Mellors found $\gamma$-globulin in the cytoplasm of two morphologically distinct types of plasma cells in man, one with and one without Russell bodies. The latter include both mature and immature plasma cells. In addition, Ortega and Mellors demonstrated $\gamma$-globulin in large and medium-sized lymphocytes and primitive reticular cells in the germinal centers of lymphatic nodules, but not in the small, mature lymphocytes at the periphery of the nodule.

We identified $\gamma$-myeloma protein, as did Vasquez, in plasma cells with abundant cytoplasm and eccentrically located nuclei. We also have found this protein to be present in plasma cells which varied greatly in size and in quantity of cytoplasm.

Similarly, $\beta_2\lambda$-myeloma proteins were found in a morphologic variety of plasma cells. Although these proteins have physicochemical and immunochemical features which differ from the 6.6 S $\gamma$-globulins, we did not distinguish any morphologic differences between plasma cells forming these two types of globulins.

Although Bence Jones proteins have been associated with malignant proliferation of plasma cells, particularly immature plasma cells, there has been to date no direct proof of their intracellular presence. Our immunofluorescent observations have indicated the presence of Bence Jones proteins in plasma cells with centrally located nuclei and scanty cytoplasm. However, Bence Jones proteins were also found in a morphologic range of plasma cells in each of the four patients studied. In the fifth patient, Bence Jones proteins were present in more typical plasma cells.

The intracellular demonstration of Bence Jones proteins, in conjunction with the evidence from the biosynthetic studies indicates that they are not breakdown products of larger serum globulins. This supports the view that these proteins are synthesized de novo in the plasma cells. In patients having myeloma proteins or macroglobulinemic macroglobulins as well as Bence Jones proteins, evidence was obtained that the Bence Jones proteins and the larger anomalous proteins were present in the same cells.

Two types of Bence Jones proteins, distinguished physicochemically and immunochemically, were noted to be present in the patients with Bence Jones proteinuria. These two groups of Bence Jones proteins appear to be similar to the two groups identified by Korngold and Putnam and their co-workers, Type I being equivalent to Type B of Korngold and Lipari and Type II
equal to their Type A. The comparison between the morphologic appearance of Giemsa-stained preparations and the immunofluorescent positive cells did not reveal any consistent morphologic differences between cells containing the Type I or the Type II Bence Jones proteins.

Immunofluorescent examinations were repeated at 1- to 6-month intervals in seven patients. The findings were the same in all cases at the time of the second test, except in one patient with Bence Jones proteinuria (D. R.). This patient had predominantly lymphoid cells on first examination (fig. 4) and predominantly plasmacytic cells at the time of the second test. None of the morphologic findings could be related to prior or current chemotherapeutic experience (table 1).

Each of the specific gamma globulin components tested in the present study was diffusely distributed throughout the cytoplasm of the positively reacting cells. This is in accord with the observations of Vasquez on the gamma myeloma proteins, Curtain, Dutcher and Fahey, Burtin, Kritzman et al., and Zucker-Franklin et al. on Waldenström's macroglobulins, Mellors et al., on rheumatoid factor and White and Coons et al. on antibody globulin. In some cells the intensity of fluorescence appeared greater in the peripheral portions of the cytoplasm. A paranuclear clear zone was not prominent in the immunofluorescent studies, although such clear zones were not uncommon on Giemsa-stained preparations. The diffuse cytoplasmic distribution of immunofluorescence indicates that specific globulin was present in the Golgi zone as well as in the greater portion of the cytoplasm containing endoplasmic reticulum.

It was generally observed that all of the malignant cells reacted with anti γ-globulin antiserum and that, therefore, they contained protein. The quantity of fluorescence, however, was not uniform for all cells and may reflect differences in the globulin content of the cytoplasm of individual cells. We did not observe the geographic division of lymph node cells between γ₁-macroglobulin-containing cells and 6.6 S γ-globulin-containing cells described by Burtin, who suggested that an aberrant, possibly rapidly metabolized γ-globulin was synthesized in the latter cells.

Intranuclear inclusions were first described in the malignant lymphoid cells of patients with macroglobulinemia. Subsequently, intranuclear inclusions were noted by Christensen and Paraskevas et al. in cases of multiple myeloma with β₂₉-myeloma proteins. Recently, Britten, Tanaka, and Brecher have found intranuclear inclusions in patients with γ-myeloma proteins or Bence Jones proteins, as well as in patients with β₂₉-myeloma proteins and macroglobulinemic macroglobulins. Immunofluorescent studies in patients with increased γ₁-macroglobulins indicated that the intranuclear material included γ₁-macroglobulins. This was confirmed in two macroglobulinemic patients in the present study. In addition, in one patient with γ-myeloma protein, positive intranuclear fluorescence was seen with specific anti-6.6 S γ-globulin antiserum. The intranuclear fluorescence in this patient appeared ring-like, near the outer margin of the intranuclear inclusion body, and corresponded to PAS-positive material seen in similar locations in smears.

The availability of a variety of antisera, including specific antisera, influenced
the interpretation of the observations made in the present study. A potent polyvalent antiserum which reacted strongly with all four classes of gamma globulins facilitated identification of the cells synthesizing the gamma globulins. With specific antisera, reacting with only one type of gamma globulin (6.6 S \( \gamma \), \( \beta_2 \alpha \), or \( \gamma_1 \)-macroglobulin), it was possible to relate specific types of gamma globulins to specific cells. The present studies demonstrate a morphologic range of cells containing gamma globulins in individual patients with malignant lymphoid or plasma cell disease and of a greater morphologic range in each group of patients. It seems reasonable to postulate that, in the normal individual, a morphologic range of cells is associated with the synthesis of each gamma globulin type. Several observations have indicated that this is true for 6.6 S \( \gamma \)-globulin and macroglobulins.\(^5,32\)

The malignant cells examined in the present study contained only a single type of large globulin, i.e., 6.6 S \( \gamma \)-globulins, \( \beta_2 \alpha \)-globulin or \( \gamma_1 \)-macroglobulins. Indirect evidence was obtained that Bence Jones proteins are synthesized, in some cases, in the same cells that are forming myeloma proteins or macrogobulins. Except for the Bence Jones proteins, the malignant cells appeared to contain only one type of protein. Additional evidence that they synthesize only one type of large gamma globulin is seen in the lowered levels of other serum gamma globulin components in multiple myeloma and macroglobulinemia. These observations support the possibility that normal plasmacytes and related lymphoid cells synthesize only one type of gamma globulin, and that the large amounts of one type of globulin seen in multiple myeloma and macroglobulinemia reflects continued production by the malignant cells of protein produced in the cell in which malignancy originated.

**Summary**

The cellular localization of 6.6 S \( \gamma \)-globulins, \( \beta_2 \alpha \)-globulins, \( \gamma_1 \)-macroglobulins and Bence Jones proteins was studied by immunohistochemical procedures in 21 patients with multiple myeloma or macroglobulinemia.

Each type of protein was identified by specific immunofluorescence in a variety of morphologic forms of malignant cells. Some cells were typically plasmacytic, some were lymphoid cells and others were immature forms. It was clear that \( \gamma \), \( \beta_2 \alpha \), \( \gamma_1 \)M, and Bence Jones proteins were not associated exclusively with a single morphologic form of malignant cell. The variety of immunofluorescent positive cells in each patient was more restricted, however, than in a group of patients with a specific protein abnormality.

In patients with anomalous proteins, all or almost all of the malignant cells were found to contain the specific anomalous protein. The malignant cells contained either \( \gamma \)-myeloma protein, \( \beta_2 \alpha \)-myeloma protein or \( \gamma_1 \)-macroglobulin in patients with these types of anomalous protein. Only one class of globulin was found in individual cells. In patients with Bence Jones proteins as the sole anomalous protein, all the malignant cells appeared to have Bence Jones protein. Where an anomalous serum globulin coexisted with Bence Jones proteins, indirect evidence indicated that the Bence Jones proteins and the larger globulin were formed in the same cells.
IMMUNOHISTOLOGIC LOCALIZATION

SUMMARIO IN INTERLINGUA

Le localisation cellular de globulinas γ a 6,6 S, de globulinas β2A, de macroglobulinas γ1, e de proteinas Bence Jones esseva studiate per methodos immunohistochimic in 21 patientes con myeloma multiple o macroglobulinemia. Omne typo de proteina esseva identificate per immunofluorescentia specific in un varietate de formas morphologic de cellulas maligne. Certes del cellulas esseva tipicamente plasmocytic, alteres esseva cellulas lymphoide, e ancora alteres representava formas immatur. Il esseva clar que le proteinas γ,β2A, γ1M, e Bence Jones non esseva associate exclusivemente con un sol forma morphologic de cellula maligne. Tamen, le varietate del cellulas con positivitate immunofluorescente in le patiente individual esseva plus restringite que in un gruppo de patientes con un anormalitate proteinic particular.

In patientes con proteinas anormal il esseva trovate que omne o quasi omne le cellulas maligne contineva specific proteinas anormal. Le cellulas maligne contineva proteina de myeloma γ, proteina de myeloma β2A, o macroglobulina γ1 in patientes con iste typos de proteina anormal. Solmente un classe de globulina esseva trovate in le cellula individual. In patientes con proteinas Bence Jones como le sol proteina anormal, omne le cellulas maligne pareva haber proteina Bence Jones. In casos in que un anormal globulina seral co-eexisteva con proteinas Bence Jones, evidentia indirecte indicava que le proteina Bence Jones e le plus grande globulina esseva formate in le mesme cellula.

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Immunohistologic Localization of Gamma-1-Macroglobulins, Beta-2A-Myeloma Proteins, 6.6 S Gamma-Myeloma Proteins and Bence Jones Proteins

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