Production of Macroglobulins in Vitro and a Study of Their Cellular Origin

By Dorothea Zucker-Franklin, Edward C. Franklin and Norman S. Cooper

The role of the plasma cell in the synthesis of γ-globulin has been readily accepted because of the excellent correlation found between the number of plasma cells in tissues, the ability to form antibodies, and the level of γ-globulin in the serum. Thus, there is a lack of plasma cells in agammaglobulinemia, an increase in plasma cells in reactive hypergammaglobulinemia, and a proliferation of abnormal plasma cells associated with the paraproteinemias of multiple myeloma. Additional support for this concept was given by the localization of 7S γ-globulin in plasma cells with specific fluorescent antibody and the production of 7S γ-globulin in vitro by plasma cells from the peripheral blood of a patient with plasma cell leukemia.

It is still undecided whether 19S γ-globulin is produced by the same cell or whether another cell is responsible for its synthesis. The occurrence of hypergammaglobulinemia of the 19S type accompanied by lymphocytosis in patients with Waldenström's macroglobulinemia and in some cases of chronic lymphocytic leukemia or lymphosarcoma suggests that the lymphocytic cell series may be implicated. In these conditions there is usually no increase in the number of plasma cells or the 7S fraction of γ-globulin, and at times an actual deficiency of normal 7S immune globulins prevails. A study of the immune response in newborn infants has lent further support to the possibility that high molecular weight antibodies do not arise from mature plasma cells. It has been observed that newborns are able to elaborate 19S antibodies to a variety of antigens at a time when few if any plasma cells can be found in their tissues. In addition, several patients with congenital agammaglobulinemia have been reported in whom high titers of 19S antibodies developed following recurrent mixed bacterial infections while the 7S γ-globulins failed to rise.

In the healthy adult the level of 19S γ-globulin is very low. Therefore, in order to investigate the cellular origin of macroglobulins, patients were studied who presented an excessive production of these serum proteins asso-
Associated with an unusual proliferation of lymphoid tissue. Accordingly, three patients (K, M, and U) with macroglobulinemia of Waldenström were selected. The diagnosis was established on clinical and morphologic grounds and by immunoelectrophoresis and ultracentrifugation of the serum. Ultracentrifugal analysis showed a large peak with a sedimentation coefficient of 19S and smaller 26S and 35S peaks which together made up 30-50 per cent of the serum proteins. Each of the proteins migrated as a homogeneous peak in the γ-globulin region on paper electrophoresis and had the immunologic and chemical properties of the 19S γ-globulin fraction. One of the proteins (U) was a cryomacroglobulin.

**MATERIALS AND METHODS**

Lymph nodes were removed from the cervical or axillary region and immediately placed into a Petri dish containing Hank's balanced saline. The capsule and connective tissue were dissected away, and the gland was teased into small pieces weighing about 25-50 mg each. Three of these pieces were placed into each of several roller tubes containing 2.0 ml of the following nutrient medium:

- **NCTC 107**
- Hank's balanced saline to which had been added a mixture of amino acids from which lysine had been omitted, \( 10^{-4} \) C\(^{14}\)-L-lysine\(^{14}\) in a concentration of 1 μc (20-25 μg) per tube, and 2000 units of penicillin
- Rabbit Serum

The culture tubes were incubated at 37°C for 24 hours, after which they were immersed in dry ice and acetone. Control tissue was not incubated but was frozen in the nutrient medium immediately upon dissection. It was kept frozen for 24 hours while the experimental tissue was incubating. From then on cultured and control tissue were treated alike. The cells were ruptured by thawing and brief homogenization in a Potter-Elvehjem homogenizer. The debris was removed by centrifugation, and the tissue extract was dialyzed for 48 hours against 0.15 M saline to eliminate free C\(^{14}\)-lysine.

It was assumed that any protein newly synthesized by the cultures would contain C\(^{14}\)-lysine present in the medium. The amount of C\(^{14}\)-labeled macroglobulin elaborated by the cultures could then be determined by precipitating it with a specific antiserum. The rabbit antiserum used for this purpose was prepared against one of the pathologic macroglobulins as described elsewhere.\(^{11}\) It was made specific for 19S γ-globulins by repeated absorption with 7S γ-globulin and gave a single line against whole human serum on double diffusion in agar.\(^{11}\) Since minute amounts of radioactive amino acids may adsorb to antigen-antibody complexes nonspecifically,\(^{12}\) the tissue extracts were absorbed twice with a diphtheria toxoid-equine antitoxin precipitate before precipitation of γ-globulin was attempted. Each time the protein content of the absorption complex was considerably larger than the final macroglobulin precipitate. The radioactivity of the precipitates was determined in a micromil window gas-flow counter. Synthesis of macroglobulins was considered to have taken place in the cultures when the radioactivity of the specific macroglobulin precipitate exceeded the radioactivity of the last absorption precipitate by more than 25 cpm.

To identify the type of cell responsible for the production of the protein, the fluorescent antibody technic\(^{13}\) was used. Frozen sections, lymph node imprints, bone marrow smears, and smear prepared from the thoroughly washed buffy coat of peripheral blood were stained with the antiserum conjugated with fluorescin isothiocyanate by a modification of

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\(^{*}\)Difco.

\(^{†}\)Nuclear Chicago Corp., Chicago.
Table 1.—Radioactivity Incorporated into Macroglobulins in 13 Tissue Cultures of Lymph Nodes from Three Patients (K, M, and U) with Macroglobulinemia of Waldenstrom

<table>
<thead>
<tr>
<th>Patient</th>
<th>Culture</th>
<th>Macroglobulin ppt. C.P.M.</th>
<th>Absorption ppt. C.P.M.</th>
<th>Difference C.P.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>1</td>
<td>118</td>
<td>8</td>
<td>110</td>
</tr>
<tr>
<td>K</td>
<td>2</td>
<td>93</td>
<td>8</td>
<td>85</td>
</tr>
<tr>
<td>K</td>
<td>3</td>
<td>95</td>
<td>2</td>
<td>93</td>
</tr>
<tr>
<td>K</td>
<td>4</td>
<td>180</td>
<td>25</td>
<td>155</td>
</tr>
<tr>
<td>K</td>
<td>5</td>
<td>197</td>
<td>53</td>
<td>144</td>
</tr>
<tr>
<td>M</td>
<td>1</td>
<td>301</td>
<td>116</td>
<td>185</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>164</td>
<td>76</td>
<td>88</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>419</td>
<td>116</td>
<td>303</td>
</tr>
<tr>
<td>M</td>
<td>4</td>
<td>143</td>
<td>42</td>
<td>101</td>
</tr>
<tr>
<td>M</td>
<td>5</td>
<td>361</td>
<td>33</td>
<td>328</td>
</tr>
<tr>
<td>U</td>
<td>1</td>
<td>100</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>U</td>
<td>2</td>
<td>146</td>
<td>8</td>
<td>138</td>
</tr>
<tr>
<td>U</td>
<td>3</td>
<td>126</td>
<td>3</td>
<td>123</td>
</tr>
</tbody>
</table>

the method of Coons. In each instance a similar slide was prepared with Wright's and Giemsa stains for comparison.

Results

Table 1 shows that all cultures incorporated C\textsuperscript{14}Lysino into macroglobulins, thus confirming that the protein is not only stored but actually produced by cells of the lymph nodes. Extracts of control tissue did not reveal any radioactive macroglobulin, nor could production of 19S \( \gamma \)-globulin by normal lymph nodes be detected by these technics. Very sensitive methods have recently shown the production of 19S \( \gamma \)-globulin by normal nodes.

The fluorescein-labeled antiserum demonstrated copious immunofluorescent staining of the frozen sections of two nodes of one of the patients (K) with a suggestion of a clonal distribution of the macroglobulin-containing cells. Treatment of companion sections with unconjugated antiserum was always able to block specific staining.

However, the thickness of tissue sections and the crowding of cells within them obscure much cellular detail, making it difficult to distinguish among cell types. The marrow smears and smears of the buffy coat of peripheral blood served to identify more precisely the cells containing the fluorescent dye. Such preparations were thin enough to allow examination of single cells. In specimens from all three patients, staining was associated primarily with large and medium-sized lymphocytes and with the cells sometimes referred to as "lymphoid reticulum" cells. Small lymphocytes usually were not stained at all by the fluorescent anti-19S globulin; occasionally, there was a suggestion of very slight staining in contrast to the brilliant fluorescence of nearby large cells (figs. 1a and 1b). In one of the subjects (K) studied during the early phase of the disease, when the peripheral blood showed a lymphocytosis consisting of mature small lymphocytes, none of the white cells in the buffy coat stained with fluorescent antibody. However, during the terminal phase of the disease, when lymphoblasts and other large pathologic-appearing leukocytes
Figs. 1 a-d.—Cells stained with fluorescein-conjugated antiserum to macroglobulin. 

a & b: Lymph node imprints; c & d: peripheral blood. a. Small lymphocyte overlying part of fluorescein-stained cytoplasm of larger cell. Autofluorescence of small cell could not be eliminated completely. b. Brightly staining cell in center appears to be medium-sized lymphocyte. c. Large lymphoblast with eccentric nucleus. Top shows nucleus of similar cell with fragments of stained cytoplasm. Large cells are very susceptible to breakage during washing of buffy coat. d. Typical fluorescein-stained medium-sized lymphocyte.

had entered the circulation, there were many fluorescein-stained cells in the peripheral blood smear (fig. 1c). This finding further supports the impression gained from the study of lymph node imprints that the large apparently immature lymphocytes produce the macroglobulins, whereas the small lymphocytes which invade the marrow and blood early in the process do not seem to play a major role.

The cytoplasm of these large cells was eosinophilic when stained with Wright's and Giemsa stains, and also gave a strongly positive PAS reaction. The nuclei presented a loosely arranged chromatin pattern and usually showed one or more nucleoli. No increase in typical plasma cells was seen in the lymph nodes or the marrow, and such cells were never seen in the peripheral blood even during the terminal phase of the disease. On fluorescence microscopy there was rarely any doubt that the stained cells represented large lymphocytes rather than plasma cells. Though the cell depicted in figure 1c had an eccentric nucleus, it was much larger in diameter than a typical plasma cell, and its nucleus showed a loose chromatin arrangement. Many similar cells were interpreted to be lymphocytes in the Wright's and Giemsa preparations of peripheral blood (fig. 2).

DISCUSSION

Evidence has been presented by several observers that cells in the marrow and lymph nodes of patients with macroglobulinemia of Waldenström and in the inflammatory tissue of patients with rheumatoid arthritis stain with fluorescein-conjugated antiserum to macroglobulin. Though this has been
interpreted to mean that the cells also synthesize the protein, definitive proof has been lacking until the present time. The findings presented in this report clearly demonstrate that a population of lymphoid cells many of which can be shown to contain 19S γ-globulin with specific fluorescent antiserum, is capable of synthesizing the protein in vitro.

The production of myeloma protein by cells concentrated from the peripheral blood of a patient with plasma cell leukemia has been demonstrated by
a method resembling ours. However, the synthesis of 7S γ-globulin by normal mature plasma cells has been established only by inference. Because the plasma cell has an ergastoplasm which fills almost the entire cytoplasm, and its electron-microscopic appearance resembles that of cells which actively synthesize and secrete protein, it appears to be well suited for the production of serum globulins. At the present time such a function is not commonly attributed to the lymphocyte or its precursors. Yet, the tissue cultures which produced macroglobulins in the experiments described in this report consisted almost entirely of cells belonging to the lymphoid series (fig. 2). Also, Ortega and Mellors reported that the large and medium-sized lymphocytes and the reticular cells in the germinal centers of lymph nodes show “some degree” of specific cytoplasmic fluorescence with an antibody to γ-globulin. Coons and Kaplan showed that fluoresceinated antigen attaches to some cells of antigenically stimulated nodes. The peripheral zones of these glands which consist of mature lymphocytes did not stain with these methods. In addition, it has been observed that the germinal centers of these nodes always contain large cells which do not stain with the antisera. Thus it would be tempting to speculate that the normal mature, small lymphocyte which has never been shown to contain γ-globulin either in our own or in previous studies arises from the large lymphocyte which fails to fluoresce. The mature plasma cell on the other hand, could conceivably be derived from the large cell in the germinal center which has been shown to contain γ-globulin. This may account for the observation by Mellors et al. who found rheumatoid factor in apparently mature plasma cells by the fluorescence technic. If one pursues this trend of thought, the morphology seen in Waldenström’s macroglobulinemia would represent a maturation arrest in the globulin producing cell line. A discussion of the particular alteration or stimulus which causes the proliferation of a macroglubulin-producing cell series is beyond the scope of this paper.

It should be pointed out that during the early phase of the disease, the non-globulin containing lymphocytes appear to mature normally, though production seems to take place at an accelerated rate. The peripheral lymphocyte count ranged between 15,000-80,000/cu. mm. in the three patients who were the subjects of this study. An alternate possibility is that the “normal” appearing lymphocytes have an abnormally prolonged life span, such as has been shown to be the case in some patients with chronic lymphocytic leukemia. The pyknotic cells which sometimes consist of nothing but denuded nuclei, frequently seen in this disease, may represent degenerated forms which have stayed abnormally long in the circulation. Terminally, when the disease becomes fulminating, immature large lymphocytes and blasts (globulin- and non-globulin containing) emerge in the peripheral blood. The lymph nodes and marrow lose their normal architecture completely and become more and more infiltrated with these large malignant appearing cells.
Chromosome abnormalities in patients with Waldenström’s macroglobulinemia\textsuperscript{2,3,3} and multiple myeloma\textsuperscript{4,1} have been reported. It would be most interesting to know at what stage of differentiation these abnormalities appear, and whether the mature lymphocyte is affected as well as the large lymphocyte, which seems to be responsible for the production of the abnormal globulin. If such studies could be correlated with the fluorescent antibody technic, the question of the origin of plasma cells on the one hand and of lymphocytes on the other might be brought one step closer to a solution.

**Summary**

Lymph nodes of three patients with macroglobulinemia of Waldenström were studied in tissue culture and shown to synthesize $19S \gamma$-globulin in vitro. Lymph node imprints, bone marrow, and buffy coat smears of the same patients consisted almost entirely of lymphocytes. When these were stained with fluorescein-conjugated antiserum to macroglobulin, large and medium-sized lymphocytes and lymphoblasts rather than mature lymphocytes or plasma cells were shown to contain the protein.

It is suggested that $19S \gamma$-globulin may also be synthesized by cells belonging to the lymphoid series under normal circumstances.

**SUMMARIO IN INTERLINGUA**

Nodos lymphatic ab tres patientes con macroglobulinemia de Waldenström esseva studiate in histocultura; esseva demonstrate que illos synthetisa globulina gamma $19S$ in vitro.

Imprimitos de nodo lymphatic, medulla ossee, e frottis de coagulo blanc ab le mesme patientes consisteva quasi completemente de lymphocytos. Quando istos esseva tinturate con fluoresceino-conjugate antisero anti macroglobulina, il esseva demonstrate que le proteina esseva presente in lymphocytos de dimensiones grande o intermedie e in lymphoblastos plus tosto que in lymphocytos matur o in plasmocytos.

Es sugerite que possibilemente globulina gamma $19S$ es synthetisate etiam sub conditiones normal per cellulas del serie lymphoide.

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Dorothea Zucker-Franklin, M.D., Assistant Attending Physician, Medical Division (Hematology), Montefiore Hospital; Fellow in Electronmicroscopy, Department of Anatomy, New York University School of Medicine, New York, N. Y.

Edward C. Franklin, M.D., Assistant Professor, Department of Medicine, New York University School of Medicine, New York, N. Y.

Norman S. Cooper, M.D., Associate Professor, Department of Pathology, New York University School of Medicine, New York, N. Y.
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