In Vitro Kinetics of Immunoglobulin Synthesis and Secretion by Nonsecretory Human Myeloma Cells

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To characterize the defect in “nonsecretory” myeloma, and to relate it to the secretory phenomenon of human myeloma cells, plasma cells from such a patient were studied in vitro and compared with other myeloma cells. The cells had the morphologic appearance of plasma cells, and contained cytoplasmic IgA-λ, but lacked surface membrane Ig and were PAS-negative. The rough endoplasmic reticulum and Golgi apparatus were anatomically intact, but the endoplasmic reticulum was distended with proteinaceous material. Incorporation of 3H-labeled leucine and carbohydrate precursors into Ig, as determined by immune coprecipitation, was measured for nonsecretory and secretory myeloma cells. Typical myeloma cells synthesized intracellular Ig, then actively secreted the newly synthesized protein into the medium. The nonsecretory cells synthesized Ig, though at a slower rate, but only released a small amount into the medium. The Ig released by these cells had a much higher 3H-carbohydrate/3H-leucine incorporation ratio than that released by the secretory myeloma cells. The data suggest that these cells have a block at the level of the Golgi apparatus, perhaps related to glycosylation, that prevents secretion. This finding may represent an abnormality induced during the malignant transformation or the clonal proliferation of a cell from a subpopulation of immunocytic precursors present normally in small numbers.

Nonsecretory myeloma, or otherwise typical clinical multiple myeloma in which no secreted paraprotein can be detected in serum or urine, occurs rarely in the spectrum of plasma cell dyscrasias. Using direct immunofluorescence, previous studies have demonstrated that monoclonal immunoglobulin (Ig) is present in the plasma cells from some cases of nonsecretory myeloma. Such intracellular monoclonal Ig has also been identified by immunoelectrophoresis of homogenates of such nonsecretory plasma cells.

The kinetics of Ig biosynthesis and secretion by mouse myeloma cells and mouse myeloma cell mutants have been extensively studied. However, these features of human myeloma cells in the usual Ig-producing variety and the uncommon nonsecretory type have not been well characterized. The intracellular mechanisms accounting for a lack of paraprotein secretion in these cases remain speculative. Possible explanations include an anatomically disordered secretory apparatus in malignant plasma cells, failure of the glycosylation that ordinarily accompanies Ig secretion, or neoplastic growth of a cell line arrested midway in the proposed transformation from lymphocyte precursor to plasma cell.

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Blood, Vol. 50, No. 6 (December), 1977 1031
In order to characterize the secretory phenomenon of such defective human plasma cells, we have studied the bone marrow and peripheral blood cells from a patient with nonsecretory myeloma and compared the kinetics of Ig biosynthesis of these cells in vitro to those from patients with the typical variety of multiple myeloma. In this report we describe our findings by light and electron microscopy, surface and direct immunofluorescence, and short-term studies of Ig synthesis and secretion in vitro.

MATERIALS AND METHODS

Case Report

B.B., a previously healthy 42-yr-old man, felt rib pain in April 1975. X-rays in June 1975 showed widespread lytic bone lesions and an open rib biopsy was interpreted as plasmacytoma. Serum protein electrophoresis showed 4.4 g/dl albumin and 0.6 g/dl gamma globulin, but no abnormal protein. His 24-hr urine protein excretion was 65 mg and a urine protein electrophoresis on a x 100 concentrated specimen showed no abnormal protein. Immunoelectrophoresis likewise revealed no monoclonal spike in the serum or urine. The hemoglobin was 9.6 g/dl and the white blood cell count was 6900/cu mm with a normal differential. The platelet count was 115,000, BUN 10 mg/dl, and calcium 12.7 mg/dl.

Treatment was initiated with BCNU, melphalan, and prednisone, with some relief of pain and return to normal of calcium. The bone x-rays did not improve, and after two months of treatment the patient was transferred to the Durham VA Hospital. Bone marrow biopsy and aspirate were obtained. Serum and urine electrophoresis and immunoelectrophoresis were repeated again, showing no abnormal protein. Quantitative Ig tests showed: IgG, 710 mg/ml (nl 640-1400); IgM, 19 mg/ml (nl 41-248); IgA, 12.5 mg/ml (nl 60-300). Treatment with melphalan and prednisone was administered, but there was a slow progression of the bone lysis over the next several months, a return of hypercalcemia, and the appearance of 1000-2000 plasma cells/cu mm in the peripheral blood. No infection occurred and the patient’s treatment was changed to Adriamycin and BCNU. The results of the cellular studies are reported below. All marrow and blood samples were obtained at least 3 wk after a course of chemotherapy.

Light Microscopy

Air-dried bone marrow and peripheral blood cells were stained with Wright’s or PAS stain using standard laboratory techniques.

Electron Microscopy

Cells and spicules of marrow were fixed at room temperature in 0.1 M sodium cacodylate buffered 3% glutaraldehyde overnight, and were postfixed in 1% osmium tetroxide in the same buffer. The tissue was then dehydrated through a graded series of alcohol and embedded in Epon. Thin sections were stained with lead citrate and uranyl acetate and were examined at 60 kV with a JEOL 100B electron microscope.

Preparation of Cells

Peripheral blood cells were collected in heparinized plastic syringes under sterile conditions. They were sedimented with plasmagel (HTI, Buffalo, N.Y.), 20°v/v and incubated for 30 min with carbonyl iron. The mononuclear fraction was collected from a Ficoll (Sigma Chemical, St. Louis, Mo.) and Hypaque (Winthrop Laboratories, New York, N.Y.) gradient as described in detail previously. Bone marrow cells were transferred immediately after aspiration into heparinized McCoy’s 5A medium (Gibco, Grand Island, N.Y.), dispersed by several passes through a 21-gauge needle, and layered over a Ficoll-Hypaque gradient. After centrifugation for 30 min at 800 g at room temperature, the resulting interface layer was collected for study. This layer contained predominantly plasma cells, but there were also some immature myeloid and erythroid cells. It was depleted of mature granulocytes, red cells, late normoblasts, and megakaryocytes.
Immunologic Studies

Immunoelectrophoresis of serum and urine in agar was conducted at pH 8.8 using commercial monospecific antisera (Meloy Labs, Springfield, Va.) of proven antigenic specificity. For determination of surface-membrane Ig by immunofluorescence, cells were washed three times with medium 199 (Gibco), supplemented with 1% gelatin, mixed with fluorescein-conjugated antisera of predetermined verified specificity (Meloy), incubated 45 min at 4°C, washed three times with medium 199, and examined in suspension with a Leitz Ortholux fluorescence microscope as previously described. For intracellular immunofluorescence studies, cells were washed five times and applied to microscope slides using a cytocentrifuge (Cytospin, Southern Instruments, Camberly, England). The slides were incubated with fluorescent antisera for 45 min at 4°C, washed thoroughly with phosphate-buffered saline (pH 7.2), and examined using a Leitz Ortholux fluorescence microscope. The specificity of all antisera was verified by immunoelectrophoresis and Ouchterlony analysis.

In Vitro Ig Synthesis

Sterile isolated peripheral blood or bone marrow cells were washed twice in either minimal essential medium without leucine (Gibco), or medium 199 without dextrose (Microbiological Associates, Bethesda, Md.) and adjusted to 5 x 10^6 cells/ml. For the determination of new protein synthesis, 3H-L-leucine, 46 Ci/mM (New England Nuclear, Boston, Mass.), was added to the leucine-free medium at a final concentration of 5 μCi/ml. For the assessment of carbohydrate incorporation into Ig, 3H-L-fucose, 10-15 Ci/μM, or 3H-D-mannose, 5-15 Ci/μM (New England Nuclear) was added to dextrose-free medium at a concentration of 10 μCi/ml. Aliquots were then incubated in a humidified 5% CO2 incubator for selected periods up to 8 hr, removed from the incubator, and centrifuged at 1200 g for 5 min, and the supernatants were collected. Cell buttons were then washed twice with phosphate-buffered saline, pH 7.0, and digested with 0.75% deoxycholate (Sigma), for 1 hr. Supernatants and cell digests were dialyzed for 48 hr at 4°C against phosphate-buffered saline, pH 7.0. The resulting dialysates were centrifuged for 10 min at 13,000 g in a Sorvall RC 2B centrifuge, and the supernatants were subsequently analyzed for labeled Ig.

Immune coprecipitation was performed by a modification of the method of Cohen and Kern. Briefly, aliquots of culture supernatants or cell extracts were incubated with carrier human Ig (polyvalent IgM, IgG, and IgA) and goat anti-human Ig (Gibco) at equivalence for precipitation. This antiserum had been previously absorbed and had reactivity against IgA, IgM, IgG, and light chains, but no other serum proteins. Simultaneously, incubations for the control of nonspecific precipitation were performed with carrier egg albumin and rabbit anti-egg albumin. The specific and control precipitates were both adjusted, at equivalence, to form 0.6 mg of precipitate so that counts nonspecifically trapped in the precipitate could be determined. Precipitation was carried out in phosphate-buffered saline, pH 7.0, in a total volume of 2 ml. Incubation was carried out at 37°C for 1 hr and then overnight at 4°C. Immuneprecipitates were washed three times with phosphate-buffered saline, pH 7.0, and solubilized with NCS tissue solubilizer (Amersham Searle, Arlington Heights, Ill.). Solubilizer was added to POPOP toluene phosphor at a ratio of 1 ml to 10 ml and radioactivity was detected with a liquid scintillation counter. Counts detected in the control, nonspecific precipitate, were subtracted from the counts incorporated into the specific Ig-anti-Ig system to give the final specific Ig counts. These background counts were generally less than 15% of the total. The system has previously been shown to detect Ig with a high degree of specificity and sensitivity. Since the antiserum and antigens employed contained all Ig determinants, including both κ and λ light chains and heavy chains, the system detected the total Ig synthesized.

RESULTS

The bone marrow was largely infiltrated by plasma cells, and similar cells were seen in the peripheral blood, comprising 95% of the mononuclear cells present at the time studied. The PAS stain was negative. Figure 1 shows the ultrastructural characteristics of the bone marrow cells. They contained exten-
Fig. 1. (A) Atypical plasma cell exhibiting deep nuclear infoldings, peripheral heterochromatin, dilated rough endoplasmic reticulum, and (top right) a prominent Golgi apparatus. Note extensive rough endoplasmic reticulum in plasma cell at bottom left. ×14,000. (B) Convoluted nucleus in a plasma cell with focally dilated rough endoplasmic reticulum cisternae. ×8000. (C) Large single-membrane–limited intranuclear inclusion in a plasma cell. The lumen is filled with proteinaceous material resembling that seen in the rough endoplasmic reticulum cisternae of A and B. ×11,000.

Sive endoplasmic reticulum, which in many cells was engorged with proteinaceous material. Russell bodies were not seen. The cells showed nuclear irregularities with nuclear infolding and single-membrane–limited nuclear inclusions as well as a well developed Golgi apparatus. Ultrastructurally, the cells were felt to be typical of plasma cells obtained from patients with plasma cell myeloma.

Bone marrow plasma cells and cells obtained from the peripheral blood during the plasma cell leukemic phase were studied for intracellular and surface Ig: 80% of the peripheral blood mononuclear cells and bone marrow plasma cells had strongly positive intracellular staining with the specific anti-IgA serum. IgG and IgM were not detected in these cells by this technique. An occasional cell was strongly positive with anti-\(\kappa\), while the anti-\(\lambda\) serum produced strong positive fluorescence in approximately 80% of the cells. On the other hand, less than 5% of these cells from either peripheral blood or bone marrow had surface immunofluorescence when stained in the viable state with anti-IgG or anti-IgM, and none with anti-IgA. Approximately the same small number of
cells also demonstrated anti-λ and -κ fluorescence. No predominance of either λ staining or IgA staining could be seen.

Figure 2 depicts the in vitro 3H-leucine incorporation into Ig by bone marrow cells of patient B.B. in contrast with that of a patient with typical IgG-secreting multiple myeloma. Thus, both the secretory and nonsecretory cells began to synthesize labeled Ig promptly, with measurable levels by ½ hr. The rate of intracellular incorporation in both cell types rose to a plateau and diminished after 4 hr. However, the secretory cells accumulated more than 15 times more intracellular labeled Ig by 8 hr than did the nonsecretory cells. 3H-labeled secreted Ig counts rose linearly in the secretory myeloma cultures and exceeded the intracellular counts by 4 hr. In contrast, in the cultures of nonsecretory myeloma cells, only a total of 400 counts/min/10⁶ cells could be detected in Ig in the extracellular fluid at 8 hr. Thus, extracellular levels never exceeded intracellular levels during this period of incubation.

Table 1 contrasts the data obtained by detecting the incorporation of 3H-leucine with that obtained by detecting the incorporation of the 3H-labeled carbohydrate precursors into Ig. After 4 hr of incubation with 3H-fucose or 3H-mannose, small amounts of radioactivity could be detected in Ig in the culture supernatant and cell extracts of patients with secretory myeloma. The
Table 1. ³H-Carbohydrate and ³H-Leucine Incorporation Into Ig (cpm/10⁶ Cells)
by Secreting and Nonsecreting Myeloma Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>³H-Leucine</th>
<th>³H-Mannose</th>
<th>³H-Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell</td>
<td>Supernatant</td>
<td>Cell</td>
</tr>
<tr>
<td>A</td>
<td>13,200</td>
<td>14,000</td>
<td>19</td>
</tr>
<tr>
<td>B</td>
<td>8,600</td>
<td>5,300</td>
<td>9</td>
</tr>
<tr>
<td>B.B.</td>
<td>2,200</td>
<td>190</td>
<td>20</td>
</tr>
</tbody>
</table>

A and B were patients with IgG-secreting myeloma. Patient B.B. was the patient with nonsecretory myeloma. Bone marrow cells were incubated with the indicated labeled precursor and extracellular supernatant and intracellular contents were separated (see Materials and Methods). The values represent the cpm incorporated into Ig/10⁶ cells after 4 hr of incubation. For ³H-carbohydrate incorporation, the supernatant and cell button of a total of 1 x 10⁷ cells were assayed because of the low counts. The total counts were then divided by 10 to express them as cpm/10⁶ cells for direct comparison with the ³H-leucine incorporation.

supernatant Ig accumulation, though at a low level, was linear during this time period with respect to secretory and nonsecretory cell incorporation of ³H-carbohydrate, with the exception of ³H-mannose for patient B, in whom there was little incorporation of this label. Despite the low level of ³H-leucine incorporation, especially into extracellular Ig, patient B.B.'s cells accumulated as much labeled mannose or fucose as did the secretory cells.

DISCUSSION

Multiple myeloma is characterized by the proliferation of malignant plasma cells, which characteristically results in the production of monoclonal Ig protein found in the serum and/or urine. Rarely (in about 1% of patients) no monoclonal protein is detectable in the serum or urine.¹² The explanation for the failure of cells from such patients to produce any Ig protein in the case of so-called “nonsecretory myeloma” is not known. Although a number of mouse myeloma cell models analogous to these types of cells have been developed, neither the developmental basis nor the intracellular mechanism for the aberrant synthetic pattern has been fully established.⁶¹³

Our patient fulfilled the criteria for nonsecretory myeloma because he had extensive bone lesions, anemia, and plasma cells in the bone marrow, but had only hypogammaglobulinemia in the serum and no monoclonal serum or urine protein abnormality. By electron microscopy these cells had all the morphologic and structural features characteristic of plasma cells, including ample rough endoplasmic reticulum, as previously seen in some nonsecretory myeloma cells.¹⁴ The cells had intranuclear inclusions similar to those previously described in myeloma cells.¹⁵ There was evidence of dilated endoplasmic reticulum by electron microscopy, but the morphologic development of the Golgi apparatus and endoplasmic reticulum appeared otherwise normal, suggesting that the lack of Ig production by these cells was not due to an anatomically disordered secretory apparatus. Rather, it would appear that the Ig molecules were unable to migrate through the Golgi, thus distending the endoplasmic reticulum and perhaps resulting in invaginations of the contiguous nuclear envelope producing the single-membrane-limited intranuclear inclusions (Fig. 1C).
Though Ig protein has previously been demonstrated within such cells and is presumably synthesized by them, actual new Ig synthesis (as determined by the incorporation of \(^3\)H-leucine into newly synthesized Ig) and the subsequent fate of such Ig has not been studied. Intracellular Ig synthesis in our patient's "nonsecretory" cells occurred in a manner analogous to that of more typical IgG-secreting myeloma cells. Ig protein was readily detectable within several minutes and reached a plateau within 4 hr of incubation. However, in striking contrast to the secreting myeloma cells, little synthesized Ig protein was ever detected in the extracellular medium of the nonsecretory cells. Moreover, the maximum level of \(^3\)H-leucine incorporation into intracellular Ig was much lower for the nonsecretory myeloma cells.

These data demonstrate that these nonsecretory myeloma cells are quite capable of Ig synthesis, but fail to secrete the synthesized material from the cell. Moreover, there must also be either feedback inhibition, such that protein synthesis is turned down to a lower rate because of the absence of secretion, or more rapid intracellular digestion and destruction of the protein, or both. We were not able to distinguish between these possibilities. Precedents for both alternatives exist among mouse myeloma cell line mutants: one variant has shown a decrease in the proportion of Ig synthesis from approximately 5\(^\circ\) to 0.5\(^\circ\) of newly synthesized protein when a mutant failing to attach light to heavy chains was produced; in another mutant failure of glycosylation occurred and increased intracellular degradation of Ig was postulated.13

Defects in Ig glycosylation have been postulated as a possible mechanism for the failure of Ig-producing cells to secrete since all Ig are glycoproteins, and some have suggested that the attachment of the carbohydrate moiety is related to the secretory process.16 Our findings support some relationship between the glycosylation mechanism and the secretory abnormality. Though most IgA myeloma cells are PAS-positive, presumably because of the high carbohydrate content of IgA,17 the nonsecretory myeloma cells, though they contain intracellular IgA, are PAS-negative, perhaps indicating a lower carbohydrate content of these IgA molecules. If a full complement of carbohydrate moieties is required for secretion, one explanation of a secretory failure might be faulty attachment of carbohydrate to some chains. Thus, perhaps only those chains with a certain minimal number of carbohydrate residues can be released or smaller fragments with a high carbohydrate content are preferentially released. This postulate could explain the relatively high carbohydrate labeling of the Ig protein released by the nonsecretory cells. Since much of the carbohydrate is added in the Golgi apparatus,18 it could be the site of such a defect. This thesis would be compatible with the electron microscopy findings. The inordinate number of cells containing IgA demonstrated among the nonsecretory myelomas (4 of 10 reported by Whicher),1 in addition to our patient, suggests that such cells may have an increased susceptibility to this sort of defect. However, one cannot generalize from this one example of nonsecretory myeloma and a variety of defects may exist in other patient's cells.

It has been postulated that mature Ig-secreting plasma cells develop, from a lymphocyte B cell derived from a bone marrow precursor, through a series of differentiation steps.5 Peripheral blood B lymphocytes are characterized by the presence of surface-membrane Ig. Thus, the cells of this patient with nonsecre-
tory myeloma are certainly not normal mature B lymphocytes in view of the absence of surface-membrane Ig, as we have demonstrated, nor do they appear to be normal functionally mature plasma cells. It is possible, therefore, that this patient's cells represent cells arrested in differentiation at a point just after the loss of surface-membrane Ig and just prior to the commencement of active Ig secretion. We do not know if this is a result of the malignant transformation or of clonal derivation from a cell usually existing only transiently in B-cell differentiation. Similar cell differentiation can be induced in vitro, and preliminary studies in our laboratory have indicated analogous cells among mitogen-stimulated lymphocytes (George and Cohen, unpublished observations). Careful kinetic analysis of the secretion pattern and surface-membrane characteristics of such cells may enable us to understand the mechanism for failures in secretion, demonstrated here, and other potential abnormalities in the differentiation pattern.

REFERENCES
In vitro kinetics of immunoglobulin synthesis and secretion by nonsecretory human myeloma cells

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