Specific Identification of Intracellular Immunoglobulin in Paraffin Sections of Multiple Myeloma and Macroglobulinemia Using an Immunoperoxidase Technique

Geraldine S. Pinkus, MD, and Jonathan W. Said, MB, ChB

Paraffin-embedded tissues of 47 cases of multiple myeloma and 4 cases of macroglobulinemia were studied by the immunoperoxidase indirect sandwich technique for identification of intracytoplasmic immunoglobulin. Specific immunoglobulin with a single heavy and/or light chain was readily detected in the neoplastic cells. Studies were performed on iliac crest bone marrow biopsies which had been fixed and decalcified using Zenker's–acetic acid solution and on tissues from various sites which had been fixed in formalin. An excellent correlation was obtained between the specific immunoglobulin detected intracellularly in the plasma cells or lymphoplasmacytic cells and the monoclonal immunoglobulin found in serum or urine of these cases. The immunoperoxidase technique represents a sensitive and specific method for identifying intracellular immunoglobulin in paraffin-embedded tissues. (Am J Pathol 87:47–58, 1977)

RECENT STUDIES HAVE DEMONSTRATED that intracellular immunoglobulin may be identified and specifically characterized in paraffin sections of multiple myeloma and lymphoreticular malignancies, using an immunoperoxidase indirect sandwich technique.[1-4] A sensitive and reliable method for accurate identification of intracellular immunoglobulin which simultaneously provides the well-defined morphologic detail afforded by fixed, paraffin-embedded tissue has extensive potential applicability in characterizing plasmacytic and lymphoid malignancies.

The objectives of this report are a) to test the specificity of the immunoperoxidase technique by evaluating cases of multiple myeloma and macroglobulinemia which may be characterized by a monoclonal pattern of intracellular immunoglobulin, with a single type of heavy chain and light chain in the neoplastic cells, and b) to determine whether the immunoperoxidase technique may be effectively applied to bone marrow biopsies which have been fixed and decalcified with Zenker's–acetic acid solution. In our laboratory the latter method of processing bone marrow biopsies provides optimal cytologic detail. In previous studies, the immunoperoxidase technique has been applied to paraffin sections of tissues which generally have been fixed in formalin or other fixatives.

From the Department of Pathology, Peter Bent Brigham Hospital, Boston, Massachusetts.

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Address reprint requests to Dr. Geraldine S. Pinkus, Department of Pathology, Peter Bent Brigham Hospital, 721 Huntington Avenue, Boston, MA 02115.

Materials and Methods

The surgical pathology files of the Peter Bent Brigham Hospital for a 17-year period from 1960 to 1976, inclusive, were reviewed. The paraffin blocks were available for 47 cases of multiple myeloma and 4 cases of primary macroglobulinemia. A total of 57 biopsies were studied (two separate specimens available in each of 4 cases, and three separate specimens in 1 case). Six biopsies were processed in 1976. The time of storage for the paraffin blocks of the remaining specimens varied from 1 to 5 years for 37 specimens (majority stored 2 to 3½ years), 6 to 10 years for three specimens, and 11 to 15 years for 11 specimens. Twenty-one specimens were initially fixed in 10% formalin. Four specimens were fixed in 10% formalin, then decalcified. The remaining 32 specimens consisted almost entirely of iliac crest needle bone marrow biopsies which were fixed and decalcified in Zenker's-acetic acid solution (20:1, v/v), generally for 4 to 6 hours, then washed in running water (about 1 hour). The biopsies were then placed in 10% buffered formalin and processed routinely.

Rabbit anti-human IgD antiserum, used only for Case 32, was obtained from Behring Diagnostics (Somerville, N.J.). All other antisera and the horseradish peroxidase–rabbit antihorseradish peroxidase soluble complexes (PAP) were purchased from Dakopatts A/S of Copenhagen, Denmark (US agent, Accurate Chemical and Scientific Co., Hicksville, N.Y.). Various control slides were used, including reactive plasmacytic proliferations and tissues from patients with multiple myeloma which had been characterized by other techniques (including tissue immunofluorescence). In the reactive processes, positive and negative plasma cells were identified in the same section with the various antisera. In the control myeloma cases, the immunoperoxidase method gave a monoclonal pattern of intracellular immunoglobulin identical with that defined by immunofluorescent studies. Also, application of various antisera [i.e., anti-κ anti-λ anti-IgG (γ-chain specific), anti-IgA (α-chain specific), and anti-IgM (μ-chain specific) antisera] to serial sections of each biopsy provided internal controls for each case.

Paraffin sections were cut at 3 to 4 μ. To insure adherence of the sections to the slides, they were warmed in an oven at 40 to 45 C for about 1 hour. Higher oven temperatures and longer heating times were not used in an attempt to minimize possible denaturation of immunoglobulin. Sections of bone marrow biopsies which had been processed in Zenker's-acetic acid solution were deparaffinized, mercuric salts were removed, and the sections were processed through the appropriate solvents to absolute alcohol. Sections of formalin-fixed tissues were deparaffinized in xylene and placed in absolute alcohol. Serial sections of each biopsy were then processed as described by Taylor.[3] Endogenous peroxidase activity was "blocked" (i.e., consumed) by incubating the sections for 30 minutes in a solution of methanolic hydrogen peroxide (1 volume of 3% aqueous hydrogen peroxide to 5 volumes of methanol). The sections were then washed with water and sequentially incubated (for 30 minutes with each reagent) with the appropriate rabbit antihuman immunoglobulin antiserum (anti-κ and anti-λ diluted 1:100, anti-IgG, anti-IgA, and anti-IgM diluted 1:40), swine anti–rabbit serum protein (1:20 dilution), and PAP (1:100 dilution). Tris buffer, 0.5 M, pH 7.6, was used for all dilutions and in the other steps as indicated. The slides were washed with Tris-saline (1 volume of Tris buffer to 9 volumes of normal saline), then placed in Tris buffer for 15 to 30 minutes after each incubation. The specific sites of antibody binding were determined with a diaminobenzidine reaction [5] which yields a deep brown color in the positive cells. The reagent for this test was prepared immediately prior to use by dissolving 6 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemicals, St. Louis, Missouri) in 10 ml of Tris buffer, followed by addition of one to two drops of 3% hydrogen peroxide. Sections were incubated with this reagent for about 5 minutes, then washed in water, counterstained with hematoxylin, dehydrated, and mounted in permount.

For cases in which the initial studies failed to demonstrate intracytoplasmic heavy chains (light chains readily detected with dilutions indicated), repeat studies were per-

formed with more concentrated heavy chain antisera (e.g., 1:20 dilution). The latter studies were performed mainly for the cases designated as light chain disease to confirm the absence of heavy chains.

Clinical charts were reviewed in all cases to verify the diagnoses and to ascertain the type of monoclonal immunoglobulin (M-component) which had been identified by immunoelectrophoretic assays of serum or urine.

Results

Results obtained by the immunoperoxidase technique in 47 cases of multiple myeloma and 4 cases of macroglobulinemia are summarized in Table 1. The monoclonal immunoglobulin (M-component) identified in serum or urine is also indicated for all cases in which this information was available. There is excellent agreement between the intracellular immunoglobulin defined by the immunoperoxidase technique and the serum or urinary M-component detected by immunoelectrophoresis. In 11 cases, the M-component or the abnormal protein present in serum or urine was not fully characterized, according to available clinical data. Biopsies for most of the latter cases had been performed 10 years or more prior to this study, and immunoelectrophoretic analyses apparently were not readily obtained. In all of these cases, however, a monoclonal pattern of intracellular immunoglobulin was identified by the immunoperoxidase method.

Each case in Table 1 represents studies performed on tissues from a single patient. In 4 cases (10, 33, 41, and 48) two separate biopsies were studied, and in 1 case (49) three separate tissues were evaluated. Identical results were obtained for all specimens of a given case.

In Case 9, two distinct M-components (biclonal gammopathy) were identified by immunoelectrophoretic techniques and were characterized as a dominant IgG component and a minor IgA component. The immunoperoxidase reaction in this case showed a predominance of plasmacytic cells with IgG heavy chains and κ light chains, and a second population of plasma cells with IgA heavy chains and λ light chains.

Cases 45, 46, and 47 apparently represent nonsecretory myelomas, as described previously by Hurez *et al.*[6] Typical bone lesions and bone marrow changes of myeloma were evident in all 3 cases. However, extensive studies revealed no serum or urinary M-component and no proteinuria. In Case 45, hypogammaglobulinemia was evident. Using the immunoperoxidase technique, all 3 cases showed a monoclonal pattern of intracellular immunoglobulin. Case 31 (IgA,K pattern) may also represent a case of nonsecretory myeloma in that multiple assays of serum and urinary protein demonstrated only "trace Bence Jones protein" on one occasion.

Table 1—Comparison of Intracellular Immunoglobulin and M-Component in Serum or Urine in Multiple Myeloma and Macroglobulinemia

Case No.	Tissue	Intracellular immunoglobulin*	M-Component in Serum* or Urine
IgG Myeloma			
1	Iliac crest, needle biopsy	IgG,K	IgG,K
2–8	Iliac crest, needle biopsy	IgG,K	IgG
9	Iliac crest, needle biopsy	IgG,K (major)	IgG (major)
		IgA,L (minor)	IgA (minor)
10	Femur, pathologic fracture	IgG,K	IgG
	Rib mass	IgG,K	
11	Humerus, pathologic fracture	IgG,K	IgG
12	Vertebral marrow	IgG,K	M-component in serum†
13	Iliac bone marrow clot	IgG,K	Hypergammaglobulinemia†
			Bence Jones proteinuria†
14	Iliac crest, needle biopsy	IgG,L	IgG,L
15–22	Iliac crest, needle biopsy	IgG,L	IgG
23	Vertebral marrow biopsy	IgG,L	IgG
24	Iliac bone biopsy	IgG,L	M-component, gamma region†
25	Sternal marrow biopsy	IgG,L	Hypergammaglobulinemia†
IgA Myeloma			
26	Iliac crest, needle biopsy	IgA,K	IgA,K
27	Iliac crest, needle biopsy	IgA,K	IgA
28	Iliac bone biopsy	IgA,K	IgA
29	Epidural mass	IgA,K	IgA
30	Epidural mass	IgA,K	IgA
31	Sternal mass	IgA,K	Bence Jones proteinuria (trace)†‡
IgD Myeloma			
32	Oral tumor	IgD,L	IgD
Light chain disease			
33	Iliac crest, needle biopsy	K	K (serum and urine)
	Iliac crest, needle biopsy	K	
34	Iliac crest, needle biopsy	K	K (urine)‡
35	Iliac crest, needle biopsy	K	K (urine)‡
36	Iliac crest, needle biopsy	K	M-component in urine†‡
37	Iliac crest marrow clot	K	K (serum and urine)
38	Clavicle	K	K (urine)‡
39	Epidural mass	L	M-component in urine†‡
40	Vertebral marrow biopsy	L	Bence Jones proteinuria†‡
41	Biopsy of iliac mass	L	Bence Jones proteinuria†‡
	Renal biopsy (tubular casts)	L	
42	Sternal marrow biopsy	L	Bence Jones proteinuria†‡
Extramedullary plasmacytoma			
43	Axillary lymph node	IgG,K	IgG
44	Soft tissue, thigh	IgA,K	IgA
Nonsecretory myeloma			
45	Iliac bone marrow clot	IgA,L	No M-component (serum or urine)
46	Femur, pathologic fracture	K	No M-component (serum or urine)
47	Iliac crest, needle biopsy	L	No M-component (serum or urine)
Macroglobulinemia			
48	Iliac crest, needle biopsy	IgM,K	IgM
	Iliac crest, needle biopsy	IgM,K	
49	Iliac crest, needle biopsy	IgM,K	IgM
	Spleen	IgM,K	
	Splenic hilar lymph node	IgM,K	
50	Iliac crest, needle biopsy	IgM,K,L	IgM
51	Iliac crest, needle biopsy	IgM,K,L	IgM

K = kappa light chain, L = lambda light chain.
* All M-components refer to serum, unless otherwise indicated.
† Not further characterized.
‡ No serum M-component.

In Cases 48 and 49 of macroglobulinemia, a monoclonal pattern (IgM,K) of intracellular immunoglobulin was found. In Case 48, the second bone marrow biopsy was obtained 5 years after the initial biopsy. In Case 49 a bone marrow biopsy, spleen, and splenic hilar lymph node all contained plasmacytic and lymphoplasmacytic cells with intracellular immunoglobulin of IgM,K type. Bone marrow biopsies in the 2 other cases of macroglobulinemia showed IgM-type heavy chains within the infiltrating cells. However, about equal numbers of cells contained either κ or λ light chains. The presence of both light chains in these cases apparently does not represent lack of specificity of the light chain antisera, in that monoclonal light chains were detected in the cases of myeloma. In addition, further studies of these cases with more dilute light chain antisera (1:200 dilution) also demonstrated significant numbers of cells with either κ or λ light chains. Comparing the clinical features of these cases of macroglobulinemia, in Cases 48 and 49, which were associated with an IgM,K pattern of intracellular immunoglobulin, the patients had a more rapidly progressive course than the patients in Cases 50 and 51 (IgM,K,L). Both patients of the former group have died. Serum IgM levels varied from 1700 to 7500 mg% (normal 50 to 200 mg%) in these 2 patients. In Case 48, 5 years after a diagnosis of macroglobulinemia had been made, the patient developed carcinoma of the lung and died shortly thereafter with widespread metastases. In Case 49, progressive deterioration was related to complications associated with macroglobulinemia. The most prominent features in Cases 50 and 51 were anemia and an extensive lymphoplasmacytic bone marrow infiltrate. Both patients are alive and have a serum M-component, with IgM levels in Case 50 which range from 1030 to 2180 mg% and in Case 51, from 244 to 450 mg%. These 2 patients have been treated with prednisone and Leukeran and have had uncomplicated courses, with follow-up times of 3 and 4 years, respectively.

Positive results obtained by the immunoperoxidase technique are assessed on the basis of deep brown staining in the cytoplasm of the neoplastic cells, i.e., at the site of antibody binding. Interpretation was readily achieved in all cases in that the neoplastic cells showed moderate to strong cytoplasmic staining only for a single heavy chain and/or a single light chain. Varying numbers of neoplastic cells demonstrated positive staining in a given case. In most instances, a majority of neoplastic cells or nearly the entire population gave positive results. Serial sections which had been incubated with antisera other than that of the heavy and/or light chain characterizing a given case were distinctly negative and generally showed only the hematoxylin counterstain. The definitive nature of these results is illustrated in Figure 1 for a case of

IgG,L myeloma. In an occasional case, a predominance of plasmacytic cells showed a single heavy and/or a single light chain, with rare or small numbers of plasma cells containing immunoglobulin with another type of heavy and/or light chain. The latter, when present, comprised a very minor proportion of the total population. In some cases of myeloma, the intercellular areas of the plasmacytic infiltrate and the "background serum" also appeared stained following the diaminobenzidine reaction, but usually less intensely so than the plasmacytic cells. Typically, this finding was noted only with the antiserum for the heavy and/or light chain which was identified intracellularly and did not appear to represent nonspecific staining. In a few cases, amyloid was also present in the biopsy and showed the same pattern of staining as the intercellular material. Some degree of background staining was observed in a small number of cases, particularly those which have been fixed in formalin and/or stored for prolonged periods of time. This finding, however, did not interfere with interpretation, in that the positive cells were much more intensely stained than the background material.

In the 2 cases (43 and 44) of extramedullary plasmacytoma, tissue immunofluorescent was also performed on frozen sections of the tumor. Results were identical to those obtained by the immunoperoxidase method. In Case 41 (light chain disease), tubular casts present in a renal biopsy showed immunofluorescent staining for λ light chains, similar to the result obtained by the immunoperoxidase assay.

Discussion

Until recently, intracellular immunoglobulin could be effectively demonstrated in tissue sections primarily by the use of immunofluorescence. Although the latter method is indeed sensitive and specific, it requires the use of frozen tissue and is unsuitable for studies of fixed, paraffin-embedded material. A further disadvantage of immunofluorescent techniques is that they do not afford optimal cytologic detail or a permanent preparation.

Immunoperoxidase sandwich techniques as described in several reports have been successfully applied to formalin-fixed, paraffin-embedded tissue for identification of intracellular immunoglobulin or other tissue antigens.[1-4,7-9] The immunoperoxidase method employs an immunoglobulin-enzyme bridge for localization of tissue antigens. The components of the bridge method basically include a) antiserum specific for the tissue antigen, e.g., for intracellular immunoglobulin; b) antiserum against the immunoglobulin of the species used for the initial antiserum; c) specific antiserum, prepared in the same species as the initial antiserum,

directed against an enzyme label (e.g., against horseradish peroxidase); and d) the enzyme label. A more sensitive, but equally specific, variation of this technique utilizes horseradish peroxidase–antihorseradish peroxidase soluble complexes (PAP).[3,10] In this study, the latter variation was employed.

The immunoperoxidase method results in a permanent histologic preparation with good morphologic detail. The accuracy and specificity of this technique as applied to detection of intracellular immunoglobulin have been demonstrated by Taylor and Mason [2] in their studies of formalin-fixed, paraffin-embedded tissues of patients with multiple myeloma. Their findings have been confirmed by our study which includes a larger series of cases of multiple myeloma and macroglobulinemia than those previously reported. Both studies show excellent correlation between the intracellular immunoglobulin detected by the immunoperoxidase technique and the M-component identified by immunoelectrophoretic methods, in the serum or urine of patients with these disorders.

In reviewing the clinical charts of the patients in our study, it was apparent that in some cases, multiple studies of serum and/or urinary protein were required to define an M-component. This was particularly true in cases of light chain disease. The immunoperoxidase method applied to the plasmacytic infiltrate in bone marrow or other tissues thus provides another type of analysis for characterization of the M-component in these cases. In other instances, several bone marrow aspirates were required for a definitive diagnosis since the plasmacytic proliferation of the initial specimen was limited and/or not sufficiently atypical. The immunoperoxidase method may also resolve this type of problem by defining a proliferation of plasma cells or lymphoplasmacytic cells as either monoclonal (neoplastic) or polyclonal (reactive) type. In the 3 cases of nonsecretory myeloma in our study, only the immunoperoxidase technique was capable of defining the immunoglobulin abnormality associated with these disorders. The intracellular immunoglobulin identified in our 3 cases of nonsecretory myeloma, respectively, was IgA,L, κ light chains only, and λ light chains only. Hurez *et al.*[6] reported 4 cases of nonsecretory myeloma which were characterized by immunofluorescent studies of bone marrow smears. In 2 cases, the plasmacytic cells contained IgA. In the other 2 cases, only κ light chains were found.

In our cases of macroglobulinemia, a monoclonal pattern of IgM,K type was found in 2 cases, while the other 2 cases showed IgM heavy chains and κ and λ light chains. A more benign clinical course has been evident in the latter 2 cases when compared with the 2 cases in which an intracellular monoclonal IgM,K pattern was noted. However, the small

number of cases evaluated and the relatively limited follow-up in Cases 50 and 51 preclude basing any firm conclusions on the pattern of intracellular immunoglobulin in cases of macroglobulinemia.

In our laboratory, optimal cytologic detail is obtained for bone marrow biopsies following fixation and decalcification in Zenker's–acetic acid solution. Figure 1 attests to the excellent histologic preparation afforded by this method. This type of processing not only provides good morphologic detail but in addition, does not interfere with subsequent determinations of intracellular immunoglobulin by the immunoperoxidase technique. To our knowledge, there are no previous reports of immunoperoxidase studies on bone marrow biopsies which have been treated by this type of preliminary fixation and decalcification.

The immunoperoxidase sandwich method represents a reliable and specific means of identifying intracellular immunoglobulin in routinely processed tissues and has extensive potential applicability. Malignant lymphomas of both Hodgkin's and non-Hodgkin's type may be further characterized by this technique. It has been suggested that the majority of nonHodgkin's lymphomas are of B-cell type and follicular center cell origin.[11] One would then anticipate that intracytoplasmic immunoglobulin with a monoclonal pattern may be identified in at least some of these neoplasms, especially those of large cell type and those with plasmacytoid features. The immunoperoxidase method is particularly applicable to this type of evaluation of malignant lymphomas, in that extensive retrospective studies of paraffin-embedded tissues may be performed. In several recent reports,[3,4] intracellular immunoglobulin of monoclonal type has been identified in some non-Hodgkin's lymphomas by the immunoperoxidase technique. Similar results have been obtained in preliminary studies of non-Hodgkin's lymphomas in this laboratory.[12]

Another area in which the immunoperoxidase method may be of value is in assessment of undifferentiated malignant tumors. Some tumors of this group probably are poorly differentiated malignant lymphomas. If intracytoplasmic immunoglobulin of a monoclonal type can be identified in such tumors, this finding would provide strong evidence for a malignant lymphoma. Studies of tissues from Hodgkin's disease by this method have demonstrated that varying proportions of Reed-Sternberg cells contain intracytoplasmic immunoglobulin, suggesting a B-cell origin for these cells.[3,4,13,14]

Application of the immunoperoxidase method to large numbers of paraffin-embedded biopsies of lymphoreticular malignancies or lymphoplasmacytic proliferations may provide valuable information in characterizing these processes. In cases of multiple myeloma and macroglobulinemia,

intracellular immunoglobulin may be specifically identified in routine biopsies by the immunoperoxidase technique.

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[*Illustrations follow*]

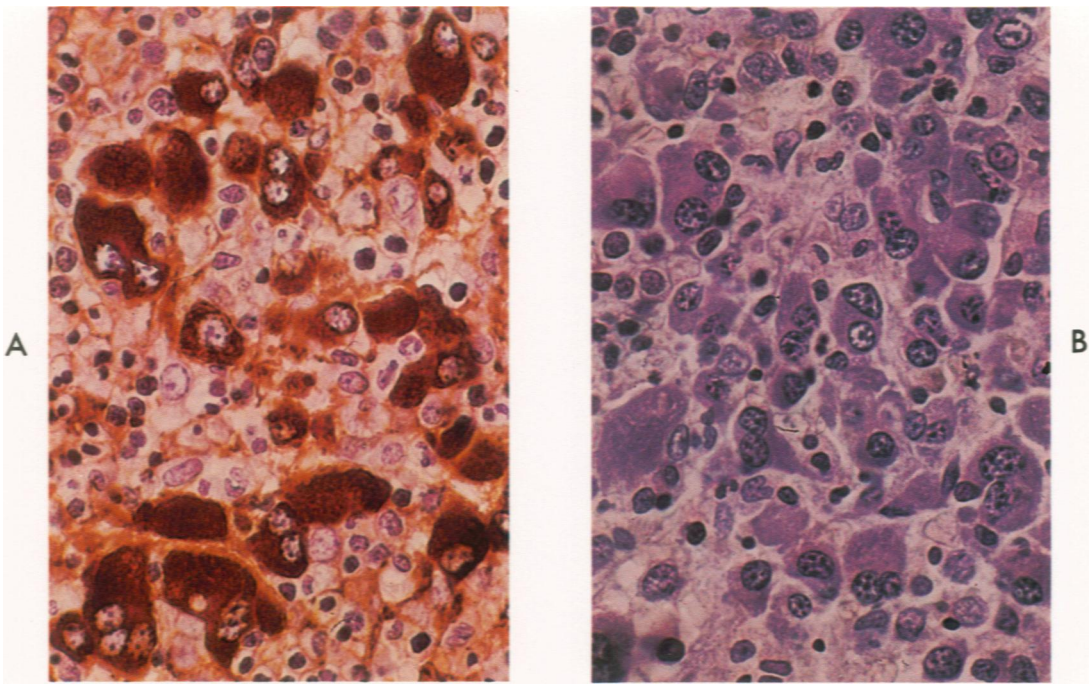

Figure 1—Multiple myeloma, IgG,L type. Iliac crest bone marrow biopsy fixed in Zenker's–acetic acid solution. Results of immunoperoxidase technique following initial incubation with rabbit antihuman λ light chain antiserum **(A)** or rabbit antihuman κ chain light antiserum **(B)** (Hematoxylin, × 450). **A**—In this section, the cytoplasm of the neoplastic plasmacytic cells appears dark brown. Hematopoietic cells present in the same field show only hematoxylin counterstain. A similar result was obtained with rabbit antihuman IgG antiserum. **B**—In this section, all cells show only hematoxylin counterstain. Similar results were obtained following incubation with rabbit antihuman IgA and IgM antisera.