Thirty-Six Human Monoclonal Immunoglobulins With Antibody Activity Against Cytoskeleton Proteins, Thyroglobulin, and Native DNA: Immunologic Studies and Clinical Correlations

By Guillaume Dighiero, Brigitte Guilbert, Jean-Paul Fermand, Peggy Lynderi, Françoise Danon, and Stratis Avrameas

Six hundred and twelve monoclonal Ig (MIg) were studied for their antibody activity against the following autoantigens: actin, tubulin, thyroglobulin, myosin, myoglobin, fetuin, albumin, transferrin, and double-stranded DNA (dsDNA). Of these 612 MIg, 36 (i.e., 5.75%) were shown to possess antibody activity. Thirty-two of these 36 (5.22% of the total) were mainly directed against actin. The four others were directed, respectively, against tubulin, myosin, thyroglobulin, and dsDNA. The interaction of the MIg with the respective antigen was demonstrated by immunoenzymatic methods with monospecific antisera and by blotting experiments. Furthermore, this interaction in the 12 cases studied was mediated by the dimeric fragment Fab of the MIg. The MIg with antitubulin, antithyroglobulin, and anti-dsDNA activities were exclusively inhibited by their homologous antigens. Those with antianticin activity were predominantly inhibited by actin and also by tubulin and thyroglobulin. The one binding to myosin was, for the most part, inhibited by myosin and also significantly by actin and tubulin. Retrospective clinical analysis was possible for 31 of 36 patients. Twenty-six of 31 had malignant lymphoplasmoctic disorders. The five others were followed for miscellaneous disorders without overt signs of multiple myeloma (MM) or Waldenström’s macroglobulinemia (WM). The correlation between the antibody activity of the MIg and the clinical features is discussed. These results indicate that a high proportion of MIg possess antibody activity against actin (5.22%). This incidence contrasts sharply with the positive reactions found toward all the other antigens tested: only one each for dsDNA, tubulin, thyroglobulin, and myosin, and none against myoglobin, fetuin, albumin, and transferrin. The significance of these results and the relationship between MIg and natural antibodies are discussed.

In a previous investigation of normal human sera, we consistently detected natural antibodies mainly directed against the following autoantigens: tubulin, actin, thyroglobulin, myoglobin, fetuin, albumin, and transferrin. On the basis of these results, a preliminary study, designed to test the antibody activity of MIg against the above antigens, indicated that a high incidence of MIg possessed antibody activity against cytoskeleton proteins, and that possibly these MIg might share antibody specificities with natural antibodies. As this study was restricted to a small number of MIg, it appeared necessary to extend it. We therefore added double-stranded DNA (dsDNA) and myosin to the seven abovementioned antigens. We also attempted to establish whether a correlation existed between the possession of antibody activity by an MIg and the clinical picture displayed by the patients.

The results of the 62 MIg already reported were added to those of the 550 studied here against the panel of 9 antigens. Of these 612 MIg, 36 (i.e., 5.75%) were shown to possess antibody activity, and 32 of these 36 (5.22% of the total) were, for the most part, directed against actin. The four others were directed, respectively, against tubulin and dsDNA (first cases reported in the literature), myosin (second reported in the literature), and thyroglobulin. Clinical data were obtained concerning 31 of the 36 patients with positive sera. Of these, 26 sera corresponded to lymphoplasmoctic disorders, and 5 to MIg with no overt signs of multiple myeloma (MM) and/or Waldenström’s macroglobulinemia (WM).

MATERIALS AND METHODS

Patient Sera

Serum samples containing 612 different MIg (273 IgG, 158 IgM, 135 IgA, 30 Bence-Jones proteins, 3 IgD, and 13 double MIg) were mostly obtained from the Department of Immunochemistry and Immunopathology of Saint-Louis Hospital (Prof. Seligmann, Paris). The homogeneous component was identified by immunoelectrophoresis with monospecific antisera (Dako Laboratories, Denmark). The level of the MIg was estimated by the total Ponceau red uptake after serum electrophoresis on cellulose acetate. All serum samples were collected between 1971 and 1981 and stored at –30°C. We excluded cryoglobulins, as well as MIg, from patients with peripheral neuropathy, as these have already been reported.

Antigens

Calf muscle actin was prepared according to Spudish and Wast and tubulin, from pig brain, according to Shelansky et al. Human transferrin was purchased from Hoechst-Behring, West Germany and human albumin from Schwartz-Mann, Orangeburg, NY. Thyroglobulin (porcine type II), myoglobin (whale skeletal muscle type II), fetuin fetal calf serum, (type III), myosin (rabbit muscle), and native dsDNA (type I from calf thymus) were purchased from Sigma, St. Louis, MO. All antigens were tested by SDS-polyacrylamide gel electrophoresis (PAGE), and no crosscontamination was found between them.
Antibodies

Rabbit antibodies to human γ, α, μ, κ, and λ immunoglobulin chains were isolated on immunoadsorbents (Institut Pasteur Production, Marnes-la-Coquette, France). β-galactosidase (Dr. A. Ullman, Institut Pasteur Fondation, Paris) was labeled to antibodies by the one-step procedure.6

Screening of Patient Sera by Enzyme Immunoassay

Polystyrene flat-bottomed NUNC plates (Denmark) were coated with antigens as previously described,7 and in the case of dsDNA, according to Labrousse et al.7 The antigen-coated plates were thoroughly washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and allowed to incubate for 2 hr at 37°C with each serum at 1/500 dilution in PBS-T containing 0.5% gelatin (PBS-T-G). A serum pool from 800 healthy donors was used as the normal reference serum. After 5 washings with PBS-T, plates were incubated for 2 hr at 37°C with β-galactosidase-labeled rabbit anti-human Ig antibody (1 μg/ml in PBS-T-G). The plates were then washed again and the enzyme substrate was added. The resultant color after 1 hr at 37°C was determined by Titertek multiskan.

Characterization of Patient Sera

The control values obtained from the serum pool were expressed as 100%. Only sera from patients exhibiting at least 15 times the normal activity were considered potentially positive and further processed using other techniques.1,2

Determination of the isotype. For this purpose, plates were first incubated with the patient’s serum and then with monospecific anti-μ, anti-γ, anti-α, anti-κ, and anti-λ antibodies that were labeled with β-galactosidase, and the enzymatic activity was measured. To confirm that binding to the antigens was mediated by the MIg, 4 sera were separated by isoelectric focusing in agarose gel.3 The fractions obtained from the gels were eluted, and the antibody activity of each one was assessed by immunoenzymatic methods, as described above.

Preparation of F(ab')2 fragments. F(ab')2 from IgG and IgA were prepared according to the procedure described by Nissonoff et al.1 and Plaut and Tomasi4 for IgM.

Immunologic detection of proteins on nitrocellulose sheets. Antigens were run through PAGE and transferred onto nitrocellulose sheets, according to Bowen.11 They were then incubated for 18 hr with MIg, and then 2 hr with 1 mg/ml peroxidase-labeled isolated anti-human monospecific anti-γ, anti-μ, anti-α antibodies at 50 μg/ml. The activity was revealed with diaminobenzidine L and H2O2.

Competitive immunoenzymatic assay. Sera diluted at 1/1000 in PBS-T-G or their F(ab')2 fragments (100 μg/ml) were preincubated for 2 hr at 37°C with the 9 antigens at decreasing concentrations (10, 3.3, 1, 0.3, 0.1, and 0.04 nmole/ml). The mixture was allowed to react with the immobilized antigens on the polystyrene plates for 18 hr at 25°C. After 5 washings with PBS-T, monospecific anti-μ, anti-γ, anti-α, anti-κ, and anti-λ labeled to β-galactosidase were added at a concentration of 1 μg/ml. Subsequent steps were the same as those described above.

RESULTS

Of 612 sera examined, 576 did not exhibit binding exceeding the normal values. The remaining 36 sera contained antibody activity against one or more antigens. One serum (IgAX) was found to bind exclusively to tubulin, one (IgMA) bound only to dsDNA, one (IgGA) only to thyroglobulin, and one (IgAx) bound chiefly to myosin and to a lesser extent to actin and tubulin. The 32 other sera (10 IgMX, 5 IgMA, 7 IgGX, 6 IgGA, 1 IgAX, and 3 IgAA) bound mostly to actin, but also constantly and significantly to tubulin and thyroglobulin. Five of these 32 sera also bound significantly to myosin.

Characterization and Demonstration of the Immunologic Nature of the MIg-Antigen Interaction

Binding Is Mediated by MIg

Antibody isotypes were determined by incubating the antigen-coated plates with patients’ sera and then with β-galactosidase-labeled anti-μ, anti-γ, anti-α, anti-κ, and anti-λ antibodies. In all cases, the antibody activity was related to Ig molecules sharing the same light and heavy isotype as the MIg. However, in the case of the patient exhibiting an antithyroglobulin activity, serum immunoelectrophoresis indicated the presence of γ heavy chain disease protein, even though antibody activity was found to be mediated by a γMIG.

In the case of the IgMA binding to dsDNA, the same isotype reacted with DNA in the immunoenzymatic assay and also by the Farr test and immunofluorescence on Crithidia lucilae. Nevertheless, a weak immunofluorescence mediated by IgGx molecules was also found. Another serum exhibited two M-components, but IgGA alone reacted with actin.

To further demonstrate that binding to the antigen was mediated by the MIg, four MIg reacting mainly with actin and one MIg reacting only with tubulin were isolated by isoelectric focusing. The electrophoretic bands were eluted and allowed to react with the immobilized antigens. In all these cases, we found antibody activity only on the MIg bands. Furthermore, three MIg binding, for the most part, to actin were studied before and after successful chemotherapy, which had greatly reduced the MIg Level. In all three cases, the decrease in MIg was accompanied by a parallel decrease in antibody activity. At the same time, polyclonal Ig increased.

Antibody Activity Was Mediated by the F(ab')2 Fragment of the MIg

We prepared F(ab')2 fragments from 9 MIg reacting with actin (6 IgG, 2 IgM, and 1 IgA) and from MIg reacting with tubulin (IgAX), dsDNA (IgMA), and myosin (IgAX), respectively. The same binding was observed with the total serum as with the F(ab')2 of the MIg. The binding was revealed by one monospecific antiserum, the anti-light-chain antibody. Conversely, the monospecific anti-Fc antibodies did not
react. This was an additional proof of the purity of the F(ab)\textsubscript{2} fragment and allowed us to eliminate any possible contamination with the intact IgG.

**Isolated M Ig Reacted With the Antigen Transferred Onto Nitrocellulose Sheets**

We studied the M Ig reacting with tubulin (IgA\textalpha) and two M Ig (1 IgA\textalpha and 1 IgG\textalpha) reacting with actin and tubulin. In the first case, a single band of 55 kd, corresponding to tubulin, was strongly stained. On the other hand, both the M Ig reacting with actin and tubulin strongly stained 43 kd and 55 kd bands, respectively, corresponding to actin and tubulin.

**Preincubation of the Reacting Antigen With M Ig Inhibited the Reaction**

Patients' sera at a dilution of 1/1000 or F(ab)\textsubscript{2} fragments of the monoclonal Ig at a concentration of 100 \(\mu\text{g}\) were studied in a competitive immunoenzymatic assay with the 9 antigens. Sera reacting with dsDNA (Fig. 1), tubulin, and thyroglobulin were inhibited only by their homologous antigen. On the other hand, sera reacting mainly with myosin or actin were inhibited by their homologous antigen and to a lesser extent by others: sera reacting with myosin were inhibited by actin and tubulin (Fig. 2); sera [or F(ab)\textsubscript{2} fragments] reacting with actin were inhibited by tubulin, thyroglobulin, and, in 2/9 studied cases, by myosin.

**Correlation of the Clinical Status of the Patients With the Presence of M Ig With Known Antibody Activity**

The disease evolution of patients whose monoclonal component had defined antibody activity was retrospectively analyzed. Each available case was checked for characteristics and development of monoclonal gammopathy and for any indication of associated pathology. Five cases, all corresponding to dominant antitubulin activity, were excluded because of insufficient data. Of the 31 others, 19 patients had a regular exploitable follow-up.

The main clinical and laboratory findings related to the patients whose M Ig displayed anti-dsDNA, tubulin, myosin, or thyroglobulin activity are listed in Table 1. The patient with an anti-dsDNA IgG had Waldenström's macroglobulinemia (WM) and pernicious anemia; both diseases were discovered simultaneously. In retrospect, no symptoms suggesting systemic lupus erythematosus (SLE) were found.

The patient with antimyosin serum M-component had IgA\textalpha MM, with the peculiarity of recurrent pyogenic cutaneous lesions. No data were available concerning thyroid gland involvement in the patient with B-cell lymphoma and antithyroglobulin activity. The patient whose serum M-component displayed antitubulin activity was characterized by cytopenia, conspicuous splenomegaly, IgA\textalpha M Ig, and lymphoplasmocytic bone marrow histology. No significant associated disease in the past was found.

The 27 case histories of patients with dominant...
antiactin autoantibody were checked meticulously for common points. Their age and sex ratio were not significantly different from those usually found with such monoclonal gammopathies: mean age 57.7 yr (range 39–77) and sex ratio (M/F) 7/4 for the IgG Mlg; 48.5 yr (range 17–77) and M/F 1/3 for the IgA; and age 61.2 (range 38–82) and M/F 2 for IgM. The χ/λ ratio was 7/6 for IgG, 1/3 for IgA, and 10/5 for IgM. The mean serum level of Mlg was 42.2 g/liter (range 23–70) for IgG, 25 (range 15–34) for IgA, and 28 (range 5–28) for IgM.

As Table 2 indicates, 22 of these 27 patients had proliferative lymphoplasmocytic disorders (10 WM, 9 MM, 2 plasma cell leukemias, and 1 plasmablastosarcoma). There were 5 cases of monoclonal gammopathy without the usual detectable proliferation [3 benign monoclonal gammopathies (BMG), 1 Hodgkin's disease, and 1 angioimmunoblastic lymphadenopathy]. Plasmablastosarcoma was diagnosed in a 17-yr-old girl with cervical polyadenopathy and cavum localization, who died less than 1 yr after diagnosis. The majority of MM cases displayed a high tumoral mass; two were initiated by acute renal failure. Two of the WM were characterized by unusual localizations (lacrymal in

<table>
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<th>Name, Sex, and Age at Diagnosis</th>
<th>Antibody Activity Against</th>
<th>Serum M-Component</th>
<th>Special Features and Associated Disease(s)</th>
<th>Follow-up Duration (yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.B. (F) 75 DNA</td>
<td>Waldenström's macroglobulinemia</td>
<td>IgMλ 38</td>
<td>Pernicious anemia</td>
<td>Unknown</td>
</tr>
<tr>
<td>H.G. (M) 59 Myosin</td>
<td>Multiple myeloma, stage IIIA</td>
<td>IgAx 46</td>
<td>Recurrent pyodermic lesions</td>
<td>3.5</td>
</tr>
<tr>
<td>L.T. (F) 68 Thyroglobulin</td>
<td>B-cell lymphoma</td>
<td>γ 10</td>
<td>Unknown</td>
<td>4.5</td>
</tr>
<tr>
<td>P.M. (M) 82 Tubulin</td>
<td>Splenic and medullary B-cell lymphoma</td>
<td>IgAx 40</td>
<td>Prominent splenomegaly</td>
<td>Death from hemorrhagic complications</td>
</tr>
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of 9 antigens, 36 exhibited antibody activity directed against actin (32/36), tubulin (1/36), myosin (1/36), thyroglobulin (1/36), and dsDNA (1/36). The specificity of this activity was established as follows.

1. The antigen binding involved the Mlg, as it was demonstrated in three different ways: (A) In all cases, binding was mediated by a single light chain and a single heavy chain isotype corresponding to those of the Mlg. (B) In the five Mlg isolated by isoelectric focusing, it was demonstrated that the only fraction exhibiting antibody activity was the band corresponding to the Mlg. (C) Finally, in three cases in which Mlg were considerably reduced after treatment, a parallel decrease in antibody activity was found.

2. In all 12 cases studied, binding was mediated by the F(ab')2 fragment of the Mlg.

3. Antigen binding of these 12 Mlg was inhibited by the homologous antigen. In the case of antiactin and antimyosin antibodies, a significant inhibition was also found with other antigens.

4. In blotting experiments, antiactin antibodies reacted not only with actin, but also with tubulin, whereas antitubulin antibodies reacted only with tubulin.

5. Furthermore, in a previous immunocytochemical study, it was demonstrated that antitubulin antibodies specifically stained tubulin paracrystals from human cultured hepatic cells, whereas antiactin antibodies simultaneously stained actin filaments and tubulin paracrystals.

Although some human Mlg are known to possess antibody activity directed against autoantigens, bacterial antigens, and hapten, the high incidence of autoantigen activity found in this investigation (5.75%), mainly against actin (5.22%), varies from earlier reports. So far, an incidence of this order has only been found for the Fc fragment of IgG and the Ii group. Furthermore, this high incidence has until now been confined to monoclonal IgM. In this study, we found 15 IgM, 6 IgA, and 15 IgG among the 36 positive sera.

This study defines new antibody activities for Mlg and reports the first cases of Mlg directed against tubulin and dsDNA. So far, only two cases of Mlg directed against thyroglobulin have been reported, one against myosin, and one against actin. Our results seem to indicate that the frequency of Mlg directed against tubulin, dsDNA, thyroglobulin, and myosin is low, since only one case of each was found among the 612 Mlg studied. The frequency of Mlg binding mainly to actin is, on the contrary, impressive (5.22%). This incidence is much higher than would be expected if the selection of clones secreting a Mlg were to occur in a random fashion. However, the implications of this observation are still indeterminate. These results may be explained by two facts: this antibody

<table>
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<tr>
<th>Clinical Diagnosis/M-Component</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
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</thead>
<tbody>
<tr>
<td>Waldenström’s macroglobulinemia</td>
<td>—</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>6</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Plasma cell leukemia</td>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Plasmablastosarcoma</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Benign monoclonal gammopathy (BMG)</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Angioimmunoblastic lymphadenopathy</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>—</td>
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**DISCUSSION**

We reported that of 612 Mlg studied against a panel of 9 antigens, 36 exhibited antibody activity directed against actin (32/36), tubulin (1/36), myosin (1/36),
activity was not systematically tested, or the methods employed were not sensitive enough. It is noteworthy that 7 MIg with antiactin activity were considered as negative for anti-smooth-muscle antibodies. In this case, the immunofluorescence method may not be adequate for MIg known to be directed against a single antigenic determinant, which may not be adequately exposed in the cryostat stomach sections usually employed to test these antibodies.

Another important point raised by these results is the widespread reactivity observed for the 32 MIg binding “mainly” to actin and for the one MIg binding “mainly” to myosin. Our immunologic studies enabled us to exclude nonspecific binding. The constant binding of MIg with antiactin activity to tubulin and thyroglobulin, and in some cases to myosin, might be due to a homology in the primary structure of these proteins. However, to our knowledge, no clear homology has yet been established between actin and tubulin, or between the latter two and thyroglobulin; however, we cannot exclude the existence of a restricted homology shared by actin, tubulin, and thyroglobulin. Riesen and Morell found an MIg with specific cross-reactivity against DNP and nucleic acid derivatives. Lafer et al. described a monoclonal antibody reacting simultaneously with DNA and cardiolipin. This reactivity was due to the presence of diester-linked phosphate groups in both antigens. Recently, monoclonal antibodies were found to react simultaneously with different classes of intermediate filaments in mice and men. On the other hand, Hannestad described an MIgG reacting simultaneously with the Fc portion of IgG and with nucleosomes. No satisfactory explanation of this cross-reaction is presently available. Structural similarities between the cross-reacting determinants could only occur at the level of gross steric configurations. The possibility has therefore been raised that the antibody-combining site might be devoid of its usual high specificity and might be visualized as a “polyfunctional” site capable of binding a number of structurally dissimilar ligands. To better define the epitope recognized by these antibodies, we are now producing antidiotype antibodies.

The main clinical and laboratory findings for the 31 patients whose M-component displayed definite antibody activity were retrospectively reviewed. Twenty-six had lymphoplasmocytic disorders: WM (11), MM (10), plasma cell leukemia (2), plasmablastosarcoma (1), and B-cell lymphoma (2). In most cases, these patients displayed a high tumoral mass and a poor prognosis. The five others had monoclonal gammopathy of undetermined significance. However, to determine whether the presence of an autoantibody activity has prognostic value requires prospective studies.

The data for the 31 patients were surveyed in order to determine whether the peculiar antibody activity of the M-component contributed to the symptomatology of the corresponding disease. No such indication was found. In particular, the 27 MIg with antiactin activity exhibited no special or common features. These results contrast with the observations in which clinical symptoms led to the discovery of several autoantibody activities of monoclonal Ig: anti-I (chronic cold agglutinin disease), anti-IgG, antilipoproteins (xanthomas), anticitroflavin (cutaneous pigmentation), and the recently reported cases of WM associated with polynucleopathy and displaying anticytoskeleton activity. In these cases, a relationship between the MIg and the polynucleopathy is suggested by the results observed after injection of MIg component in mice. In the patient whose M-component showed antinative-DNA activity, no clinical or biologic features suggestive of systemic lupus erythematosus (SLE) were retrospectively found. However, the follow-up was not long enough to rule out clinically quiescent serologically active SLE. Another explanation may be afforded by the IgM isotype of the MIg, since IgG class antibodies are reputed to play a more important pathologic role than IgM. Another interesting point concerning this patient is the association of WM with pernicious anemia. Such an association has been reported in a few cases of MIg, but the relationship needs to be established.

The anti-smooth-muscle antibodies correspond to antibodies directed against cytoskeleton proteins. These antibodies have been related to diseases like chronic active hepatitis, infectious mononucleosis, and other acute viral illnesses. We attempted past detection of such diseases in the patients. Only two demonstrative cases were found: an active chronic hepatitis in the course of a Hodgkin’s disease and an angioimmunoblastic lymphadenopathy associated with an MIg with antiactin activity (first case cited in the literature).

We also checked each case report for indications suggestive of underlying autoimmune disorders. Besides the patient with WM, anti-native-DNA MIg, and pernicious anemia, there were only two suggestive cases: two BMG with antiactin activity.

However, the absence of underlying immunological diseases in most patients is not surprising in view of our previous results with normal serum, in which we consistently found natural antibodies. Normal natural antitubulin and antithyroglobulin appeared to be specific and were only inhibited by their homologous antigens; on the other hand, normal natural antiactin antibodies reacted strongly with actin and were specifically inhibited by actin and also by tubulin and thyro-
globulin. These antibody specificities appear to be closely related to those found for MÎµ. This would imply that, at least for some patients, the MÎµ produced might reflect the expansion of a clone producing a natural antibody. Until now, it has been assumed that a virgin B cell is first driven to clonal expansion by antigenic stimulation, and that subsequently, an oncogenic stimulus may result in neoplastic proliferation of these plasma cells. Assuming that certain malignant monoclonal gammopathies arise from the proliferation of a clone normally producing a natural antibody, it may be postulated that this is caused by the dysfunction of the system that normally regulates such antibody production.

ACKNOWLEDGMENT

We thank J.-C. Brouet, K. Delligati, and C. Mihaesco for helpful discussion. We are grateful to Muriel Bargis, Thérèse Dangui, Michèle Gilbert, and Yvette Signoret for excellent technical assistance. We also wish to thank Geneviève Limeul for the skillful typing of this manuscript.

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