APMIS 108: 764–70, 2000
Printed in Denmark . All rights reserved

APMIS
ISSN 0903-4641

Autoantibody activity of the immunoglobulin secreted by a follicular B-cell lymphoma

Y. BARRIOS,[1] A. PLAZA,[1] R. CABRERA[2] and F. DÍAZ-ESPADA[1]

Departments of [1]Immunology and [2]Hematology, Hospital Clínica Puerta de Hierro, Madrid, Spain

Barrios Y, Plaza A, Cabrera R & Díaz-Espada F. Autoantibody activity of the immunoglobulin secreted by a follicular B-cell lymphoma. APMIS 2000;108:764–70.

In patients with B-cell lymphoma, only in rare cases a secreted paraprotein is found, and in very few of them an associated autoantibody activity has been demonstrated. Here we report the case of a patient with a low-grade B-cell lymphoma with a serum biclonal paraprotein (G,M)λ and severe erythroblastopenia. Indirect immunofluorescence studies of total serum revealed cytoplasmic (Hep-2 cells) and extracellular matrix (rat tissue sections) staining, suggestive of a new specificity. After gel filtration of serum samples, only the IgM-containing fraction showed the same pattern of staining. Tumor-derived hybridomas expressed an unmutated V3–11 gene identical to that found in tumor samples and secreted an IgM immunoglobulin endowed with the same reactivity, which confirms the tumoral origin of the tissue-reactive protein. The results suggest a link between the autoimmune condition in this patient and the novel specificity displayed by the tumor-derived immunoglobulin.

Key words: Biclonal lymphoma; autoantibodies; VH-genes.

*Y. Barrios del Pino, Department of Immunotechnology, Lund University, P.O. Box 7031, S-220 07 Lund, Sweden. e-mail: yvelise.barrios@immun.lth.se

Previous work in $CD5^+$ B-CLL patients has demonstrated a high frequency of natural autoantibody activity associated with secreted monoclonal Igs (1, 2), a finding that agrees with the postulated contribution of the $CD5^+$ B1 cell subset to the synthesis of natural polyspecific IgM autoantibodies. These antibodies are an important component of the normal B-cell repertoire. A clear association between paraneoplasic symptoms and the autoantibody activity of secreted idiotypes in B-cell malignancies has not been demonstrated, although the improvement of clinical manifestations after the initiation of treatment aimed to eradicate the tumor suggests a relationship between the two conditions (3). The role that $CD5^-$ B2 lymphocytes, the predominant cell type found in follicular lymphomas, plays in natural autoantibody production is less clear. Antibodies in sera of patients with this condition or secreted by tumor-derived hybridomas have been shown to exhibit a broad specificity reminiscent of that attributed to natural autoantibodies (4, 5), but no direct relationship has been firmly established between the tumor idiotypic protein and the presence of autoimmune pathologies.

We here report a patient affected by a severe erythroblastopenia and a B-cell follicular lymphoma with biclonal IgM,G λ in serum. The serum IgM fraction was endowed with a broad tissue reactivity. Hybrids obtained by somatic fusion of tumor cells and the K6H6/B5 heterohybridoma were shown to express a V_H gene identical to that of the tumor cells and secreted an IgM protein which exhibited identical antibody specificity. The relevance of these findings is discussed.

* Present address: Department of Immunotechnology, Lund University, Sweden.

Received August 22, 2000.
Accepted October 9, 2000.

MATERIALS AND METHODS

Patient

A 38-year-old man with generalized enlargement of the lymph nodes (LN) and splenomegaly was diagnosed as having a centrocytic-centroblastic non-Hodgkin's lymphoma (Working formulation). The patient was later included in a protocol of vaccination against own tumor idiotypic protein. Microscopic examination of the bone marrow showed a decrease in red cell precursors with no signs of neoplastic infiltration. Laboratory studies showed normal parameters except for hematocrit (23%), hemoglobin concentration (7 g/dl with negative Coombs' test) and EPO (352 mU/ml). After several rounds of polychemotherapy and autologous transplantation with peripheral blood precursors, a recurrence of the tumor with $CD5^-$ $CD19^+$ $CD20^+$ $CD22^+$ $CD38^+$ DR^+ κ^- λ^{+low} cells in bone marrow, coexisting with no red cell precursors, was evidenced 2 years later. During the studies performed before implementation of the anti-idiotypic therapy, a search for ANA antibodies revealed an unexpected pattern of staining (see below). At this stage, immunofixation electrophoresis disclosed a serum biclonal paraprotein (G,M)λ, impeding the initiation of anti-idiotypic therapy. The patient died from septic shock after a severe infection.

Antibody studies

The serum of the patient or supernatants from IgM-secreting hybridomas were tested by indirect immunofluorescence (IIF) using either 6 μm frozen sections of rat kidney, liver and stomach composite blocks or cultured larynx carcinoma Hep-2 cells (Kallestad, MN, USA) and ATCC skin fibroblasts CCD-45SK. Irrelevant monoclonal Igs were used as negative control.

The IgM and IgG molecules present in the serum were separated by size exclusion chromatography (Superosa 12, FPLC). The fractions were analyzed for total protein (A280) and IgM or IgG content by a class-specific enzyme-linked immunosorbent assay (ELISA). The relevant fractions containing the monoclonal proteins were studied by IIF.

Immunoprecipitation was carried out as described

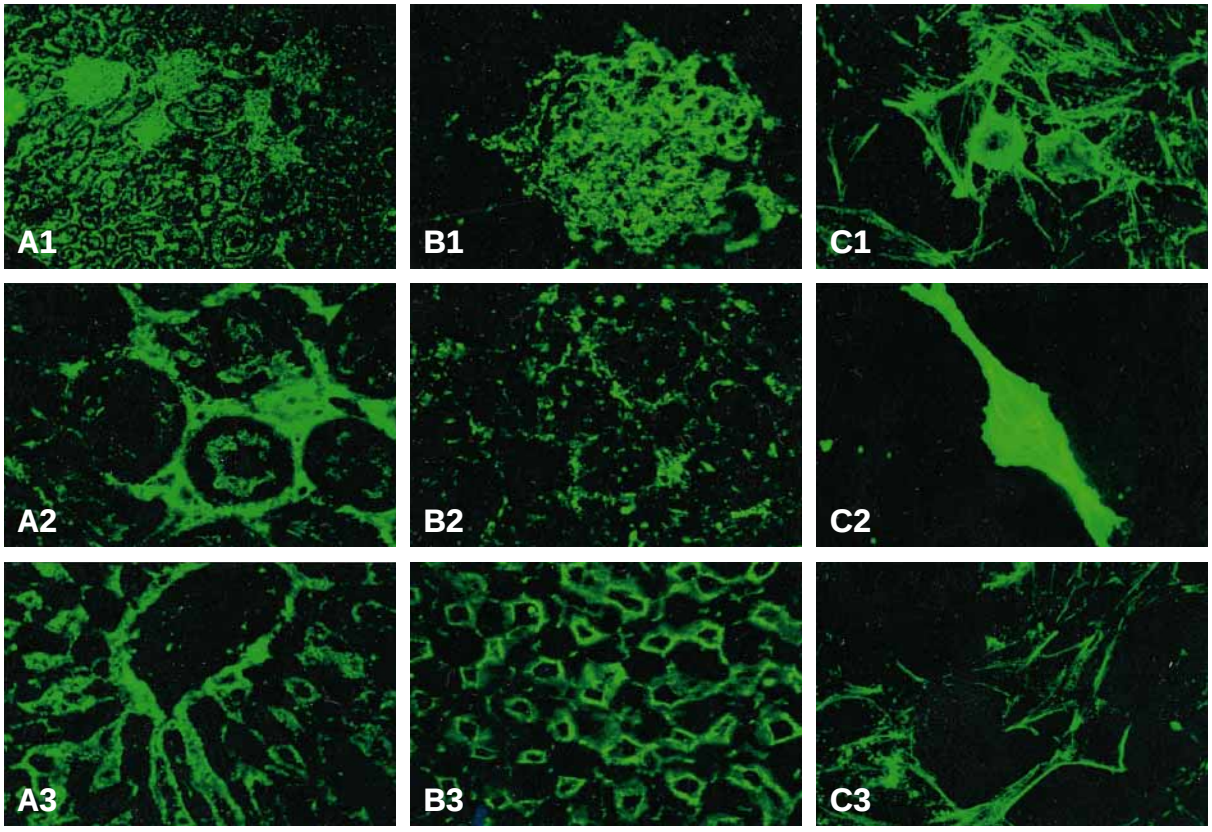

Fig. 1. Indirect immunofluorescence of patient's serum (A1-3; C1-2) or supernatant from the 2E4 hybridoma (B1-3; C3) on: A1-2/B1, rat kidney tissues; A3/B3, liver rat tissue; C1/3, Hep-2 cells, and C2, skin fibroblasts (CCD-45SK cells). See text for details.

(6). Briefly, HeLa cells were metabolically labeled with ^{35}S-methionine, resuspended in 0.05% NP-40, 50 mM NaCl, 50 mM Tris pH 7.4 containing a cocktail of protease inhibitors, and sonicated at 50 KHz. Supernatant from 10,000 rpm was mixed with protein A-agarose (Pharmacia Biotech, Uppsala, Sweden) beads previously treated with either normal or patient's serum. Anti-pl7 serum was used as internal control. The IgM component expressed by the tumor belongs to the V_H3 family (see results) and is readily absorbed onto protein A (data not shown). After washing, the beads were incubated with the labeled HeLa cell extract. Precipitated proteins were analyzed by 8% SDS-PAGE and visualized by autoradiography.

Isolation of idiotypic protein from tumor cells

Single cell suspensions from a supraclavicular LN were dispersed in RPMI medium and lymphoid cells were isolated by density-gradient centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). LN cells were fused in 50% polyethylene glycol (Boehringer Mannheim, Mannheim, Germany) with the HAT-sensitive heterohybridoma K6H6/B5 (ATCC, CRL 1823) and distributed in 96-well microtiter plates. Ig secretion was studied by conventional class-specific ELISA.

Sequence analysis of the tumor and hybridoma VH genes

Total RNA was extracted from 1×10^6 lymph node cells or 0.5×10^6 hybridoma cells (S.N.A.P., Invitrogen, CA, USA). cDNA synthesis was performed using AMV reverse transcriptase and oligo(dT) primer (Promega, WI, USA). This cDNA was amplified using a panel of V_{Hback}/J_{Hfor} family-specific oligonucleotide primers pairs (7) that expanded a single-band PCR product of about 400 bp. This product was subcloned into pCR^{TM}II using the TA cloning system (Invitrogen). The recombinant plasmids showing indentical DNA insert lengths were sequenced using a Cy5^{TM} AutoCycle^{TM} Sequencing Kit (Pharmacia Biotech) on an ALFexpress^{TM} Automater DNA Sequencer. The sequences of several clones were compared using the PCGENE program v6.85 and the search for homologous germline sequences was done using the V BASE Directory of Human V Gene Sequences (MRC for Protein Engineering Centre, Cambridge, UK).

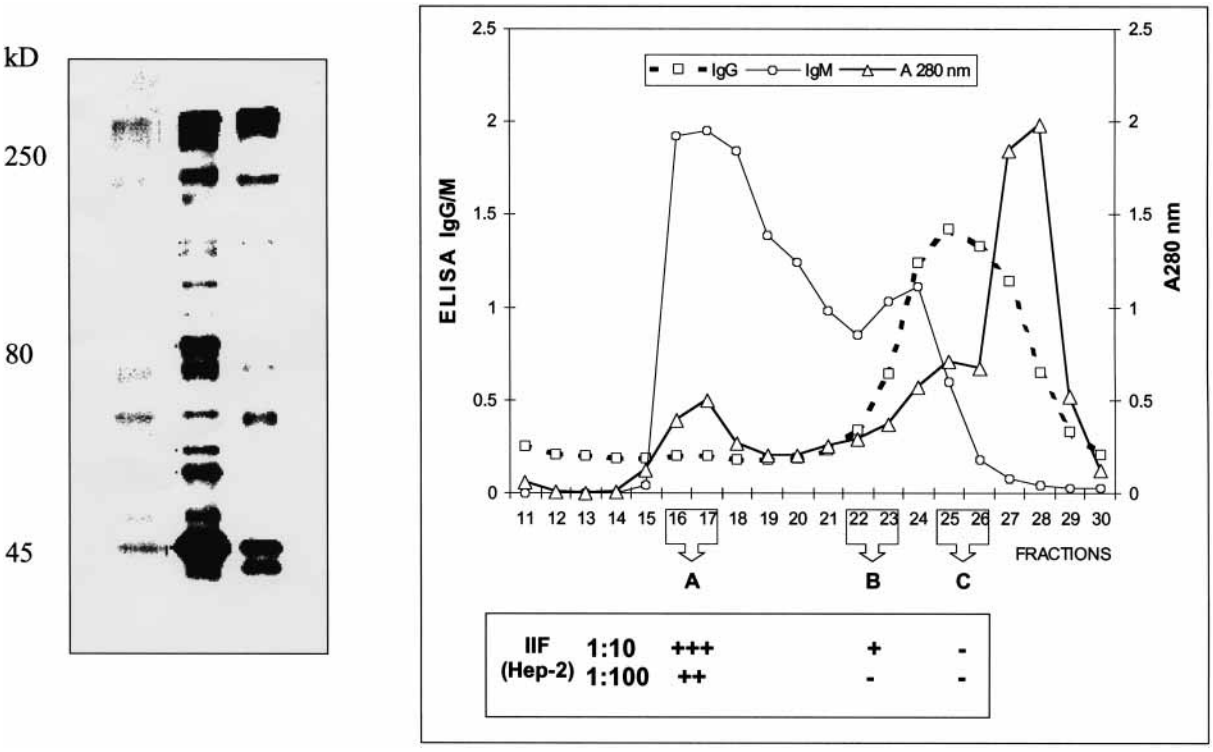

Fig. 2. Left: Immunoprecipitated proteins from methionine-labeled HeLa cell extract using (from left to right): normal human serum, patient's serum, and anti-pl7 serum (included as internal control for immunoprecipitation). Right: Profile of patient's serum chromatography on a (1×30) Superose 12 column, showing the total protein (A280 nm), IgM and IgG content (ELISA) of the collected fractions. Pooled fractions A, B and C were studied by IIF on Hep-2 cells at two different dilutions (inset).

```
         Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Leu
VH.TUM   GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTC AAG CCT GGA GGG TCC CTG
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    CDR1
         Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr Tyr Met Ser Trp
VH.TUM   AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT GAC TAC TAC ATG AGC TGG
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

         Ile Arg Glu Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Tyr Ile Ser Ser Ser
VH.TUM   ATC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTT TCA TAC ATT AGT AGT AGT
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                  CDR2
         Gly Ser Thr Ile Tyr Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg
VH.TUM   GGT AGT ACC ATA TAC TAC GCA GAC TCT GTG AAG GGC CGA TTC ACC ATC TCC AGG
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

         Asp Asn Ala Lys Asn Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
VH.TUM   GAC AAC GCC AAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    CDR3
         Thr Ala Val Tyr Tyr Cys Ala Arg Glu Ser Tyr Tyr Tyr Tyr Tyr Tyr Gly Met
VH.TUM   ACG GCC GTG TAT TAC TGT GCG AGA GAG TCT TAT TAC TAC TAC TAC TAC GGT ATG
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

         Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
VH.TUM   GAC GTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
VH.2E4   ... ... ... ... ... ... ... ... ... ... ... ... ...
VH.2G9   ... ... ... ... ... ... ... ... ... ... ... ... ...
```

Fig. 3. Nucleotide sequences of VH region of the tumor Ig and 2E4 and 2G9 hybridomas. Amino acid sequence and CDR regions (underlined) are shown. Dots indicate nucleotide identity. Conserved amino acids are in bold letters.

RESULTS

Pattern of reactivity of patient's serum

Indirect immunofluorescence studies of the patient's serum on a composite block of rat tissues showed an interstitial speckled pattern on glomeruli, intertubular areas and vessels of the kidney section (Fig. 1: A1,2) and liver portal areas (Fig. 1: A3) reaching a titer of 1:5120. In cell lines, IIF staining appeared as a fine filamentous intracytoplasmic weft, with a few scattered granules (Fig. 1: C1, Hep-2; C2, CCD-45SK). Nuclei were not stained in any of the tissues studied. Autoradiography of the immunoprecipitated proteins from ^{35}S-methionine-labeled HeLa cell extract using patient's serum (Fig. 2, left: lane 2) showed prominent 45 and 82 kDa bands and several minor specific 50, 60–65 and 135 kDa bands.

In order to assess the possible association between this pattern of staining and the biclonal component present in the patient's serum, we performed chromatographic separation of the IgM and IgG immunoglobulins by gel filtration on Superosa 12 (Fig. 2, right). The main IgM and IgG peaks containing the monoclonal material as revealed by agarose gel electrophoresis (not shown) were further

analyzed by IIF. The sample corresponding to the monoclonal IgM (fraction 16–17, A) showed a pattern of staining identical to that found using total serum, whereas the sample containing the monoclonal IgG (fractions 25–26, C) showed no reactivity (Fig. 2, right, inset).

Antibody activity of tumor-derived idiotype protein

To determine the tumoral origin of the autoantibody activity associated with the serum paraprotein, we rescued the idiotypic tumor Ig by fusion with the K6H6/B5 heteromyeloma. Thirteen out of fifteen growing hybrids secreted an IgM,λ immunoglobulin. IgG was not secreted by any of the cell hybrids tested. Cell-free supernatants from IgM secreting hybridomas were tested in IIF and showed a pattern of staining identical to that previously described (Fig. 1: B1-3,C3).

V_H gene expression in hybrids and tumor cells

The identity of the immunoglobulin derived from the IgM-secreting hybrids and tumor cells was confirmed by sequencing the expressed VDJ region. Samples of DNA were amplified with mixes of V_{Hback} and J_{Hfor} primers, cloned, and sequenced. The results of sequencing the clones revealed the involvement of primers for V_{H3} and J_{H6} families in the PCR amplification. The entire VDJ region of both tumor and hybridomas was identical except for one synonymous change at codon 104 (TAC→TAT; Tyr) in the CDR3 region of the 2E4 hybridoma (Fig. 3). A search in the VBase directory revealed that the heavy chain belongs to the VH3 family, exhibiting only one nucleotide mismatch to the V3-11 germline gene sequence. The D segment DLR1 is used in reading frame 3.

DISCUSSION

One of the most common immunological abnormalities found in patients with lymphoma is a monoclonal peak in serum (8). In this paper we report the case of a patient with a follicular B-cell lymphoma who presented a serum biclonal (M,G)λ. The serum IgM fraction reacted against a variety of cells and tissues. Idiotypic material was isolated after cell fusion and proved to be identical with that found in the tumor samples by sequencing the expressed VH genes. The IgM secreted by the hybridoma cells showed the same pattern of reactivity.

Although autoantibody activity of serum IgM in a patient with a concomitant lymphoma has been reported before (9), it has only been described in a few cases in biclonal gammapathies (10, 11) with no evidence of associated malignancy. To our knowledge, there are no previous reports showing autoantibody activity associated with individual components in a biclonal gammapathy, as it is shown in this report. In our case, the biological significance of the associated B-cell lymphoma and the lack of reactivity of the IgG paraprotein is difficult to discern. One possibility is the coexistence of two unrelated tumors in this patient, only one of which secreted an IgM autoantibody. In fact, natural autoantibodies belong almost exclusively to the IgM isotype. If this was the case, a second clone of a different kind or present at a different anatomical localization could secrete the serum IgG paraprotein. The concomitant presence of two different B-cell malignancies comprising both myeloma and NHL tumors (12, 13) or two different kinds of lymphoma has been described (14). A similar condition could account for the failure to achieve IgG-secreting hybrids that were otherwise easily obtained by us after fusion of IgG^+ B-lymphoma cells (15). Alternatively, some progenitor cells of a single original parental IgM^+ clone could have undergone class switching to IgG-secreting cells. Differences in avidity could explain the preferential binding of the pentameric low-affinity IgM (a hallmark of natural autoantibodies). On the other hand, a decrease in overall avidity after Ig class switching has been reported (16).

Although several tumor-derived molecules can be responsible for the development of paraneoplasic symptoms (17), an obvious candidate for that role is the immunoglobulin secreted by B-cell tumors. However, there is no clear demonstration that paraproteins related to natural antibodies are directly involved in autoimmune pathologies. Moreover, these antibodies are thought to be encoded for by unmutated VH genes, whereas antibodies implicated in autoimmunity exhibit somatic mutations. In our case, we did not find any other physiological

explanations for the profound erythroblastopenia found in this patient. The presence of a factor directed against EPO (18) can be discarded in the light of the high serum levels of this hormone. Given the wide distribution of the antigen recognized by the IgM monoclonal protein, most cells in bone marrow smears were stained (data not shown), impeding a direct evaluation of lineage-specific reactivity. However, any of these findings can exclude the possibility that the antibody produced by the tumor reacts either with a particular component of erythrocyte precursors or with a specific stromal cell or matrix component required for normal development of the erythroid lineage.

The pattern of staining in IIF does not correspond to any of the classic patterns associated with a particular autoimmune disease. The presence of abundant cytoplasmic filaments in the cell lines studied recalls the patterns usually associated with cytoskeletal proteins (5). Furthermore, the interstitial pattern shown in tissue is quite similar to others described in sera containing anti-high molecular weight cytoskeletal protein autoantibodies, reactive with epitopes present in the extracellular matrix, although in the only published case no association with any specific pathological condition was described (19). The fact that cytoskeletal proteins are usually present as heteropolymers fits well with the complex pattern observed in the patient's serum immunoprecipitates using metabolically labeled extract of HeLa cells. Moreover, we also found negative results when the patient's serum was tested against a panel of antigens commonly used to assess polyreactivity (tetanus toxoid, IgG, ssDNA and thyroglobulin) (data not shown).

The finding that the IgM secreted by hybridomas was endowed with the same reactivity as the monoclonal IgM in serum suggests that they are derived from the patient's tumor cells. We confirmed the identity between the tumor idiotypic protein and the product of the hybridoma cells by sequencing the expressed VH genes. Not surprisingly in a natural autoantibody, the nucleotide sequence is identical to that of a germinal V_H gene (V3-11). The V3-11 gene is expressed in fetal tissues (20) and in some variants of non-Hodgkin lymphoma (21) and encodes a significant fraction of the natural antibodies that bind to the gal α (1, 3) gal epitope (22).

In conclusion, by using somatic fusion methodology we succeeded in demonstrating the tumoral production of the autoantibody and in reproducing in vitro the behavior previously observed with the patient's serum. New approaches recently described for the identification of novel human antibody-antigen pairs (23) would help us further dissect the role of antibodies in the pathogenesis of autoimmune diseases and better understand the linkage between cancer and autoimmunity.

This work was supported by grants from Fondo de Investigaciones Sanitarias, Spain (FIS 95/1294) and Fundación LAIR. The authors are grateful to J. Hammel for technical assistance with the IIF studies and M. Messman for help in preparing the manuscript.

REFERENCES

1. Borche L, Lim A, Binet JL, Dighiero G. Evidence that chronic lymphocytic leukemia B lymphocytes are frequently committed to production of natural autoantibodies. Blood 1990; 76:562–9.
2. Mayer R, Stone K, Han A, Bona C. Malignant CD5 B cells-biased immunoglobulin variable gene usage and autoantibody production. Int Rev Immunol 1991;7:189–203.
3. Polliack A, Lugassy G. Autoimmunity and autoimmune syndromes associated with and preceding the development of lymphoproliferative disorders. Leukemia 1992;6(suppl 4):152–4.
4. Kourtis AP, Boussiotis VA, Lymberi P, Pangalis GA. Increased natural antibody activity in sera of patients with malignant non-Hodgkins lymphomas containing paraproteins. Am J Haematol 1994;46:283–8.
5. Dighiero G, Hart S, Lim A, Borche L, Levy R, Miller RA. Autoantibody activity of immunoglobulins isolated from B-cell follicular lymphomas. Blood 1991;78:581–5.
6. Matter L, Schopfer K, Wilhelm JA, Nyffenegger T, Parisot RF, De Robertis EM. Molecular characterization of ribonucleoprotein antigens bound by antinuclear antibodies. A diagnostic evaluation. Arthritis Rheum 1982;25:1278–83.
7. Hawkins R, Zhu D, Ovecka M, Winter G, Hamblin TJ, Long A, et al. Idiotypic vaccination against human B-cell lymphoma. Rescue of variable region gene sequences from biopsy material for assembly as single-chain Fv personal vaccines. Blood 1994;83:3279–88.

8. Levy R, Miller RA. Therapy of lymphoma directed at idiotypes. J Natl Cancer Inst Monogr 1990;10:61–8.
9. Nobuoka A, Sakamaki S, Kogawa K, Fujikawa K, Takahashi M, Hirayama Y, et al. A case of malignant lymphoma producing autoantibody against platelet glycoprotein Ib. Int J Hematol 1999;70:200–6.
10. Ilyas AA, Willison HJ, Dalakas MC, Whitaker JN, Quarles RH. Identification and characterization of gangliosides reacting with IgM paraproteins in three patients with neuropathy associated with biclonal gammapathy. J Neurochem 1988; 51:851–8.
11. Tschirhart DL, Kunkel L, Shulman IA. Immune hemolytic anemia associated with biclonal cold autoagglutinins. Vox Sang 1990;59:222–6.
12. Nagamura F, Goto S, Iseki T, Saotome T, Takeshita A, Kikuno K, et al. Molecular evidence for a single clonal origin in a patient With multiple myeloma and non-Hodgkin's lymphoma. Rinsho Ketsueki 1995;36:1182–7.
13. Hayakawa M, Asano T, Nakajima F, Tamai S, Nakamura H. Simultaneous presentation of malignant lymphoma of the kidney and multiple myeloma of the bone marrow. Int J Urol 1998; 5:374–6.
14. Fend F, Quintanilla-Martinez L, Kumar S, Beaty MW, Blum L, Sorbara L, et al. Composite low grade B-cell lymphomas with two immunophenotypically distinct cell populations are true biclonal lymphomas. A molecular analysis using laser capture microdissection. Am J Pathol 1999; 154:1857–66.
15. Alvarez-Vallina L, Plaza A, Kreisler M, Cabrera R, Fernández MN, Díaz-Espada F. Isolation of tumor-derived immunoglobulin-idiotype from peripheral blood mononuclear cells in a B-cell lymphoma patient with minimal disease. J Immunotherapy 1995;17:194–8.
16. McHeyzer-Williams MG, Nossal JV. Clonal analysis of autoantibody-producing cell precursors in the pre-immune B cell repertoire. J Immunol 1988;141:4118–28.
17. Broxmeyer HE, Jacobsen N, Kurland J, Mendelsohn N, Moore MAS. In vitro suppression of normal granulocyte stem cells by inhibitory activity derived from human leukemia cells. J Natl Cancer Inst 1978;60:497–511.
18. Casadevall N, Dupuy E, Molho S, Tobelem G, Varet B, Mayeux P. Autoantibodies against erythropoietin in a patient with pure red cell aplasia. N Engl J Med 1996;334:630–3.
19. Castell M. Cytoskeletal autoantibodies. In: Peter B, Shuenfeld Y, editors. Autoantibodies. Amsterdam: Elsevier Science, 1996:217–24.
20. Kraj P, Rao SP, Glas AM, Hardy RR, Milner EC, Silberstein LE. The human heavy chain IgV region gene repertoire is biased at all stages of B cell ontogeny, including early pre-B cells. J Immunol 1997;158:5824–32.
21. Braeuninger A, Küppers R, Strickler JG, Wacker H, Rajewsky K, Hansmann M. Hodgkin and Reed-Sternberg cells in lymphocyte predominant disease represent clonal populations of germinal center-derived tumor B cells. Proc Nat Acad Sci USA 1997;94:9337–42.
22. Kearns-Jonker M, Swensson J, Ghiuzeli C, Chu W, Osame Y, Starnes V, et al. The human antibody response to porcine xenoantigens is encoded by IGHV3-11 and IGHV3-74 IgVH germline progenitors. J Immunol 1999;163:4399–412.
23. Ditzel HJ, Masaki Y, Nielsen H, Farnaes L, Burton DR. Cloning and expression of a novel human antibody-antigen pair associated with Felty's syndrome. Proc Nat Acad Sci USA 2000; 97:9234–9.