Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 446-450, January 1982
Cell Biology

Human monoclonal IgM with autoantibody activity against intermediate filaments

(immunofluorescence microscopy/myeloma proteins/vimentin/Waldenström macroglobulinemia)

K. DELLAGI*, J. C. BROUET*, J. PERREAU†, AND D. PAULIN†‡

*Laboratoire d'Immunochimie et d'Immunopathologie (Institut National de la Santé et de la Recherche Médicale U 108), Institut de Recherche sur les Maladies du Sang, Hôpital Saint-Louis, 75010 Paris, France; and †Département de Biologie Moléculaire, Institut Pasteur, et Université Paris VII, 25 Rue du Dr. Roux, 75015 Paris, France

Communicated by François Jacob, September 21, 1981

ABSTRACT Monoclonal IgMs from two patients with Waldenström macroglobulinemia were found to react with intermediate filaments. This was shown by (*a*) immunostaining of various tissues and cultured cells and (*b*) immunological characterization of the reactive antigen after blotting of polypeptides separated from total cell extracts by gel electrophoresis or purified intermediate filaments on nitrocellulose sheets. One monoclonal IgM had an activity directed only against vimentin, whereas the other reacted with four different classes of intermediate filaments—vimentin, desmin, glial fibrillary protein, and keratins. All of the reactivity of the latter IgM was absorbed by purified vimentin, suggesting that different classes of proteins of intermediate filaments share common antigenic determinant(s). The significance of such autoantibody activity of human monoclonal IgM is discussed in the light of the startling frequency of IgM anti-intermediate filaments antibodies in various diseases.

Waldenström macroglobulinemia (WM) is characterized by a malignant proliferation of lymphoid and plasmacytic cells which secrete monoclonal antibodies of the IgM class (1). Monoclonal immunoglobulins from patients with multiple myeloma or WM in some instances exhibit an antibody activity directed against various auto- or heteroantigens (2, 3). Although some of these activities may reflect the immune history of the host, most recognized specificities appear to be autoantibodies. We describe here new specificities carried by two monoclonal IgMs from patients with WM that react with intermediate filaments (IF) of human or other mammalian cells.

The cytoplasm of eukaryotic cells contains filamentous proteins organized into at least three distinct networks. Two of these, microtubules and microfilaments, are composed of tubulin and actin, respectively, and these molecular structures are highly conserved throughout the species. In contrast, IF are made of at least five classes of polypeptides which differ by their biochemical characteristics, immunologic specificities, and cell type distribution (reviewed in ref. 4). Desmin [50 kilodaltons (kDal)] is found in muscle cells and myotubes (5). Glial fibrillary acidic protein (GFAP; 50 kDal) is expressed in a class of glial cells, the astrocytes (6). Three polypeptides, 200, 170, and 70 kDal, are found in neurofilaments characteristic of mammalian neurons (7). Keratin polypeptides constitute a family of proteins because at least 10 different subunits are expressed in various epithelia (8, 9). Finally, vimentin (56–58 kDal) is found not only in mesenchymal derivatives, such as fibroblasts and osteogenic, myoblastic, and glial cells (10), but also in established cell lines, in addition to specialized IF (11).

The antibody specificity of the two monoclonal IgMs reported here was established using two techniques: (*i*) immunofluorescence staining of human, mouse, and rat tissues and cultured mouse cells; and (*ii*) immunodetection of antigenic polypeptides after blotting of total cell extracts or purified IF proteins. One of the IgMs (IgM DUV) bound selectively to vimentin; the other (IgM GON) reacted with all IF except the three neuronal polypeptides. These results suggest that, in addition to class-specific antigens, different IF have some common antigenic determinants.

MATERIAL AND METHODS

Isolation of Monoclonal IgM. Each monoclonal IgM_{κ} (IgM GON and IgM DUV) was purified by ammonium sulfate precipitation and Sepharose 6B chromatography. The preparations used were devoid of contaminants as assessed by immunodiffusion at a concentration of 10 mg/ml. Fab and Fc fragments were obtained by trypsin digestion according to Plaut and Tomasi (12).

Cell and Tissue Staining. For immunofluorescent staining, 4-μm-thick sections were obtained from tissues frozen in liquid nitrogen (rat liver, kidney, striated or smooth muscle, tongue, sciatic nerve, and cerebellum, and human nerve and kidney). Sections were stained unfixed or fixed [5 min in cold acetone (−20°C) with fluorescein-conjugated monoclonal IgM (at a concentration of 50 μg/ml) or unconjugated IgM and fragments with a second layer of either rhodamine-conjugated $F(ab')_2$ fragments of rabbit IgG specific for human heavy and light chains or fluoresceinated goat IgG antibodies to human immunoglobulins.

Rat embryonic fibroblasts, mouse 3T6 fibroblasts, mouse keratinocytes, and mouse myoblasts were grown overnight in culture on coverslips and fixed for 5 min in cold acetone or methanol before fluorescent staining. In some experiments, colchicine (20 μg/ml) or cytochalasin B (10 μg/ml) was added to the culture medium for 16 or 2 hr, respectively (13).

In some experiments, the monoclonal IgMs were absorbed with purified cardiac actin or vimentin-enriched cytoskeletal material extracted from mouse fibroblasts. The insoluble filaments were homogenized by sonication and added to 200 μl of the monoclonal IgM in phosphate-buffered saline (at a concentration of 20 μg/ml) and the mixture was incubated for 6 hr at 4°C. After centrifugation, the supernatant was studied as above. The activity of control sera containing high titer of unrelated (antinuclear) antibodies was not modified by similar absorptions whereas actin or vimentin antibodies were totally absorbed only by their respective antigens.

Immunological Detection of Proteins on Nitrocellulose Sheets. Polypeptides from different cell extracts were separated by slab gel electrophoresis according to Laemmli (14) (15%

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: WM, Waldenström macroglobulinemia; IF, intermediate filaments; kDal, kilodalton(s); GFAP, glial fibrillary acidic protein.
‡ To whom reprint requests should be addressed.

acrylamide; 0.1% $NaDodSO_4$) with the discontinuous Tris·HCl buffer system. Two gels were run in parallel; one gel was stained and the other was used for transfer to nitrocellulose paper. Unstained acrylamide gels were covered with a sheet of nitrocellulose paper; polypeptide transfer was obtained after a 3 hr electrophoresis (40 V) in Tris glycine buffer (pH 8) with 20% methanol (15).

Nitrocellulose sheets were first saturated for 1 hr at 37°C in 3% serum albumin/0.2% Tween 20 in phosphate-buffered saline and then incubated for 18 hr at room temperature with the monoclonal IgM at a concentration of 100 μg/ml in phosphate-buffered saline containing 0.1% Tween 20. After extensive washing in the saline/Tween solution, the nitrocellulose sheet was incubated with peroxidase-conjugated goat IgG antibodies to human μ chains (Institut Pasteur Production). After three washes, peroxidase activity was revealed with H_2O_2 (0.03%) in the presence of diaminobenzidine (0.5 mg/ml in 0.1 M Tris·HCl at pH 7.6).

Cell Lines. The culture conditions and characteristics of the cell lines have been reported (16).

Cell Extracts and Isolation of IF. Approximately 1×10^8 cells were extracted with buffer A (0.15 M NaCl/1% Triton/10 mM Tris·HCl, pH 7). The pellet was treated with DNase I in the presence of 2 mM Mg^{2+}. The reaction was stopped by dilution with buffer B (1.5 M KCl/1% Triton X-100/10 mM Tris·HCl, pH 7) followed by three washes in the same buffer. The pellets were washed in buffer C (1% Triton X-100/10 mM Tris·HCl, pH 7) (17). Purified vimentin was obtained by elution of the 56 kDal band from acrylamide gels after electrophoresis of fibroblast IF.

Affinity Column-Purified Antibodies. The rabbit antiserum to neurofilament was prepared against a $NaDodSO_4$-denatured 70-kDal polypeptide from bovine brain (18). The vimentin antibodies were obtained by immunizing with $NaDodSO_4$-denatured vimentin from mouse 3T6 fibroblasts (19). The keratin antibodies were prepared against keratin subunits isolated from mouse keratinocytes (20). All these antibodies were made monospecific by affinity chromatography on homologous antigens coupled to Sepharose. The rabbit anti-GFAP antiserum was a gift from D. Delpech (Centre Henri Becquerel 76000 Rouen, France).

RESULTS

Monoclonal IgM DUV and IgM GON React with Fibroblast IF. Indirect immunofluorescence labeling of rat embryonic fibroblasts by the two monoclonal antibodies resulted in the staining of a filamentous network that extended throughout the cytoplasm. Direct staining with fluorescein-conjugated IgM could be observed at a concentration as low as 2 μg/ml. When cultured fibroblasts were pretreated with colchicine for 16 hr, all the stained filaments were redistributed into a perinuclear coil as is characteristic of vimentin filaments. Treatment of the cells with cytochalasin had no effect on the staining pattern, indicating that there was no reaction with actin filaments (Fig. 1). The antibody nature of the binding of the monoclonal IgM was confirmed by the reactivity of its Fab fragment, contrasting with the absence of reaction of the Fc fragment even at high concentration (2 mg/ml). Similar staining was observed when the indirect immunofluorescence procedure was performed with the purified IgM or the native sera and only with the anti-μ and anti-κ antisera. Although both IgMs had an antibody activity directed to IF, they reacted with different antigenic determinants because staining by one conjugated IgM was not blocked by the other.

Monoclonal IgM DUV Reacts with Vimentin. Sections of tissues and cultured cells were incubated with IgM DUV and stained with fluorescent anti-μ sera. In the kidney, glomerulus and arterial walls were stained. In nervous tissues, the Bergmann fibers of the molecular layer of the cerebellum and the Schwann cells of peripheral nerve were stained (Fig. 2; Table 1). These tissues are known to express vimentin (4, 11). Similarly, established cell lines that are known to express vimentin filaments showed typical staining. After treatment with colchicine (20 μg/ml), the IgM stained a coiled perinuclear ring characteristic of vimentin filaments.

In order to characterize the proteins that reacted with IgM DUV, extracts from fibroblasts, myoblasts, myotubes, cultured keratinocytes, and neuronal cells were separated on 15% acrylamide gels containing 0.1% $NaDodSO_4$, transferred onto nitrocellulose sheets, and then incubated with IgM DUV (100 μg/ml). Binding of IgM was revealed by the immunoperoxidase technique (Fig. 3). One polypeptide band of 56 kDal was strongly stained in extract from 3T6 fibroblasts. A band of the same size was detected in all cultured cell types but with various intensities, strong in extracts from myoblast clone T984 and very faint in keratinocyte clone T4. Similar experiments with partially purified vimentin gave the same results. Monoclonal IgM without known antibody activity were unreactive in this assay.

Finally, indirect immunofluorescence staining of tissues and cell lines was completely abolished by previous absorption of

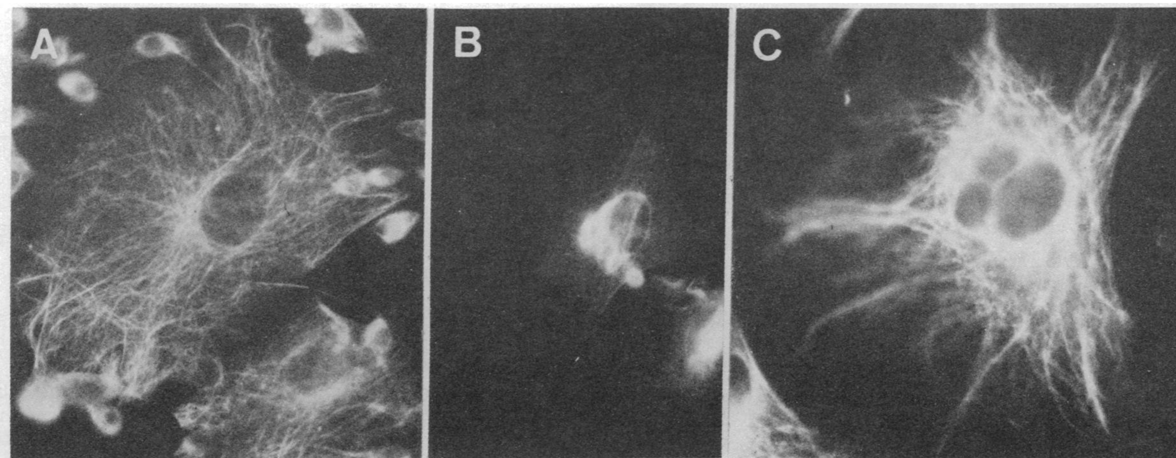

FIG. 1. Fibroblasts stained with the two monoclonal IgM. (×600.) (*A*) Typical pattern of IF revealed by IgM DUV and IgM GON. (*B*) Colcemid treatment of the cells resulted in a coiling of vimentin fibers around the nucleus of the fibroblast with IgM DUV and IgM GON; this is typical of vimentin filaments. (*C*) Cytochalasin treatment had no effect on the staining pattern.

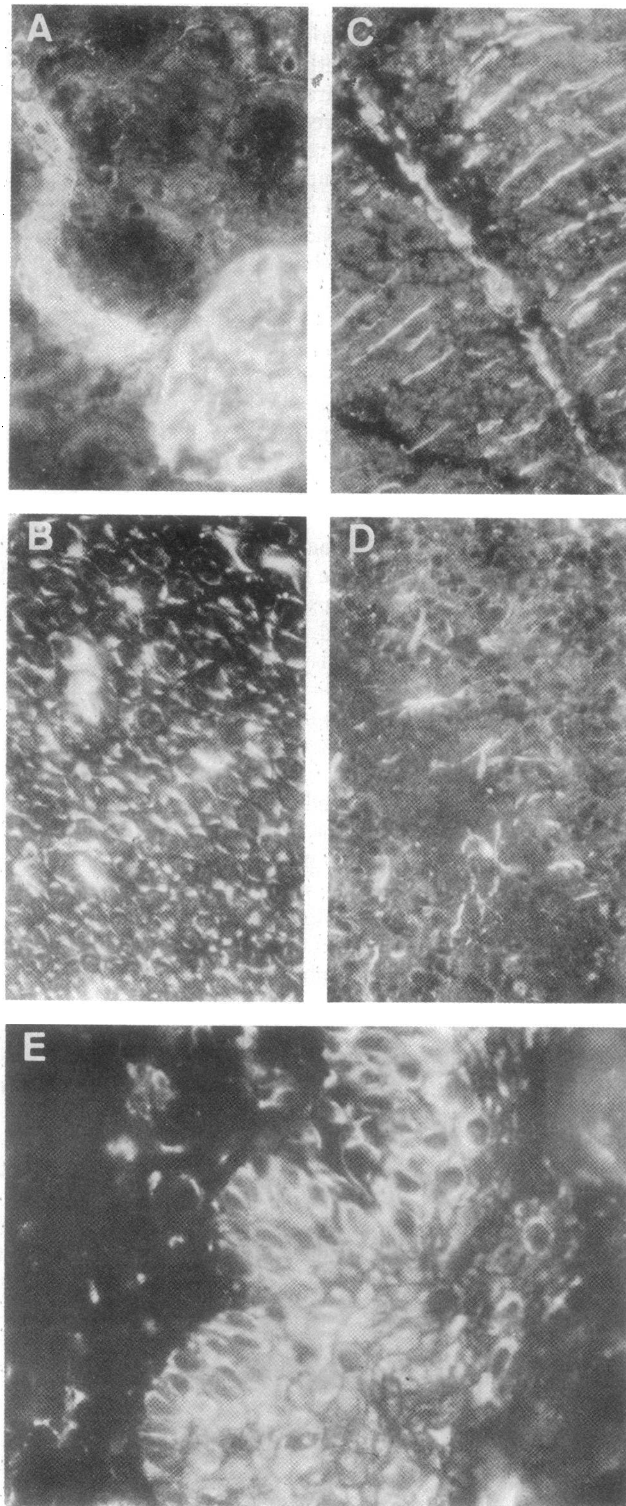

FIG. 2. Immunofluorescence studies on unfixed sections of rat tissues. (×100). IgM DUV and IgM GON had identical staining patterns on glomerulus and arteries (*A*), peripheral nerve (*B*), and the molecular layer of the cerebellum (*C*; Bergmann fibers are stained). Only IgM GON stained the granular layer of the cerebellum (*D*; astrocytes are stained) and tongue epithelium (*E*).

IgM DUV with purified vimentin.

Monoclonal IgM GON Reacts with Different Tissues and Cultured Cells. On tissue sections, IgM GON reacted with most of the tissues examined, whatever their embryonic derivation (Table 1). Of particular interest was the staining of epi-

Table 1. Tissue reactivity of IgM DUV and IgM GON as demonstrated by indirect immunofluorescence

Tissue	IgM DUV	IgM GON
Kidney:		
Glomerulus	+	+
Arterial wall	+	+
Liver:		
Hepatocytes	−	+
Epithelium:		
Tongue	−	+
Esophagus	−	+
Muscle:		
Striated muscle	−	+
Smooth muscle	−	+
Cerebellum:		
Bergmann fibers	+	+
Astrocytes of granular layer	−	+
Peripheral nerve:		
Axons	−	−
Myelin sheet	−	−
Schwann cells	+	+

thelial cells from esophagus and tongue, or liver hepatocytes, and of smooth and striated muscle. In the nervous system, the staining was restricted to Schwann cells, astrocytes of the granular layer of the cerebellum, and Bergmann fibers. No staining of neurons was observed. These findings suggested that the monoclonal IgM GON might react with IF of different classes.

Three mouse cell types previously characterized for the expression of various classes of IF were studied. The myoblastic clone T984 is able to differentiate *in vitro* to produce myotubes which express desmin polypeptide as the major constituent of IF. When myoblasts were incubated with IgM GON (or IgM DUV), a typical filamentous network, similar to that obtained with sheep or guinea pig polyclonal IgG against vimentin (19), was revealed (Fig. 4). IgM GON was also reactive with myotubes, indicating a reactivity with desmin, whereas IgM DUV was negative.

Clone T4 of keratinocytes is known to express several subunits of keratin in addition to vimentin (20). IgM GON showed a typical array of filaments associated with desmosomal junctions known to be tonofilaments. In contrast, IgM DUV stained

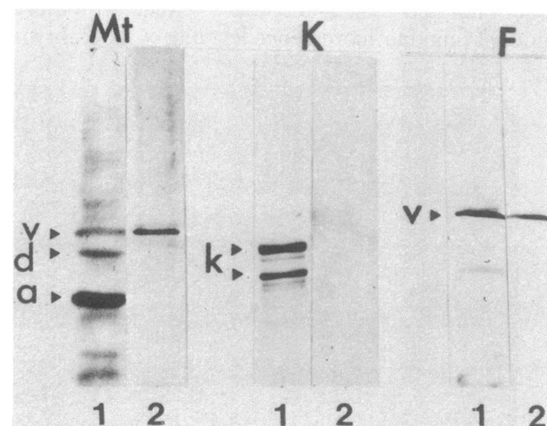

FIG. 3. Immunodetection of polypeptides binding IgM DUV after gel electrophoresis and blotting. Cytoskeletal material enriched in IF was prepared from mouse myoblasts and, myotubes (Mt), keratinocytes (K), and fibroblasts (F) and polypeptides were separated by gel electrophoresis. After transfer to nitrocellulose paper sheets, IgM DUV specifically bound vimentin from muscle and fibroblast extracts. Lanes: 1, Coomassie blue staining; 2, immunoperoxidase reaction. v, Vimentin; d, desmin; a, actin; k, keratins.

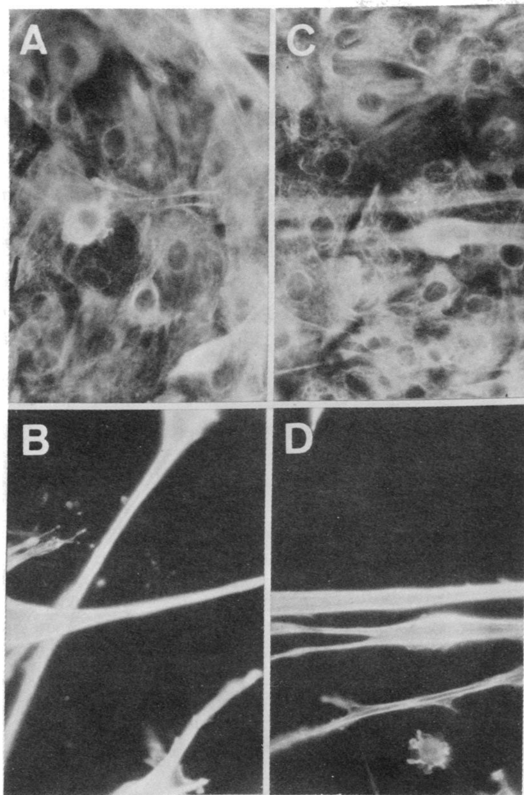

FIG. 4. Different staining patterns of myogenic cells with IgM DUV and IgM GON. (×200.) The myogenic cell population is composed of myoblasts and myotubes. (*A*) Staining of myoblasts with IgM DUV: myotubes are not stained. (*B*) Same field as in *A*, showing myotubes stained with antiactin coupled to rhodamine. (*C*) Staining of both myoblasts and myotubes with IgM GON. (*D*) Same field as in *C*, showing myotubes stained with antiactin coupled to rhodamine.

only a few fibrils, a result that correlates with the expression of only low levels of vimentin.

IgM GON did not stain neurons cultured from 3-day mouse mesencephalon with any of the fixation methods used (methanol or 3.7% formaldehyde followed by methanol). In contrast, all the glial cells were decorated.

Monoclonal IgM GON Reacts Against Four Classes of IF. In order to define more precisely the different polypeptides that react with IgM GON, immunodetection was performed as described above. Cytoskeletal material enriched for a given class of IF was analyzed on polyacrylamide gels and, after transfer to nitrocellulose paper sheets, was incubated with GON antibody. IgM GON reacted with the major bands of keratins, with muscle desmin, with vimentin (Fig. 5), and with GFAP (data not shown).

In addition, a positive reaction was found for a 66-kDal protein present in the various total cell extracts and in partially purified IF (Fig. 5). No reactivity was detected against the polypeptides of the neurofilament triplet.

These results suggested that IgM GON reacts with four different classes of proteins—namely, vimentin, desmin, keratins, and GFAP. Therefore, IgM GON was absorbed with vimentin, either in liquid phase or on vimentin coupled to Sepharose. Both procedures removed all the reactivity of IgM GON. Under similar conditions, rabbit keratin antibodies were not retained on the vimentin-Sepharose column.

DISCUSSION

The monoclonal IgMs from two patients with WM exhibited an antibody activity directed against IF. The IgM from one patient

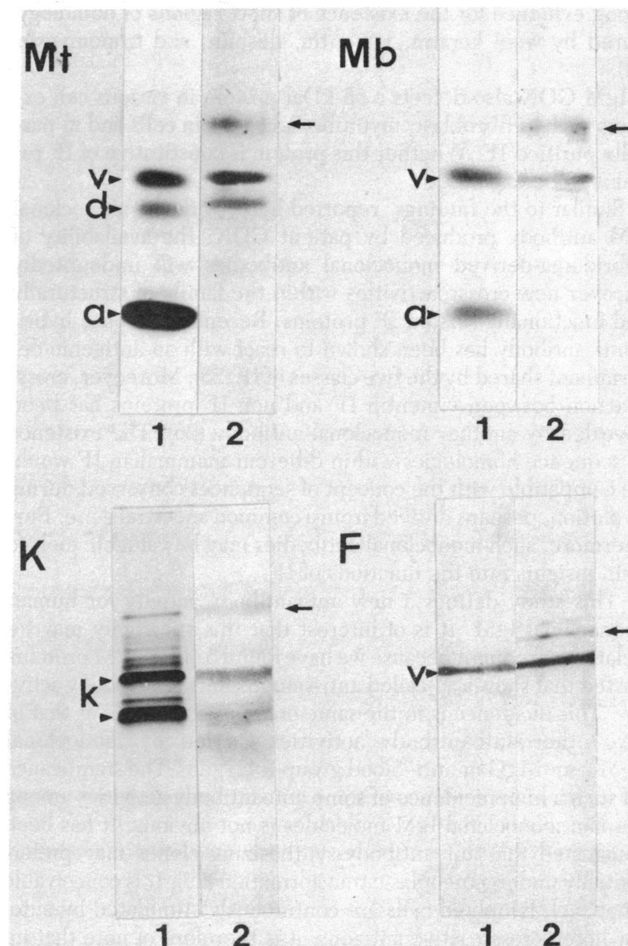

FIG. 5. Immunodetection of polypeptides binding IgM GON after electrophoresis and blotting. IF from mouse myotubes (Mt), myoblasts (Mb), keratinocytes (K), and fibroblasts (F) were analyzed by slab gel electrophoresis and stained with Coomassie blue (lane 1). Parallel gel slabs with unstained polypeptides were used for protein transfer to nitrocellulose paper sheets and incubation with IgM GON (lane 2). Two bands reacted in extracts from myotubes, corresponding to vimentin (v) and desmin (d). One major band of vimentin reacted in extracts from fibroblasts and myoblasts. Two major bands contained keratins (k). Note also an additional positive band, most clearly seen in cytoskeletal preparations from myotubes and fibroblasts, at about 66 kDal (arrow). a, Actin.

(IgM DUV) was directed exclusively against vimentin, the major polypeptide subunit that constitutes fibroblast IF. By immunofluorescence microscopy, this IgM reacts only with tissues or cells that express vimentin and it stains a single 56-kDal protein from cell extracts as well as purified vimentin. By the same criteria, the reactivity of the second IgM (IgM GON) is more widespread and involves at least four different classes of IF—namely, GFAP, vimentin, desmin, and keratins. This reactivity is totally abolished after incubation with pure vimentin. This finding suggests that different classes of IF share common antigenic determinants. The crossreactivity we have observed may be due to homology in the primary structure of these proteins. The evidence accumulated to date suggests that the proteins of the five known classes of IF have some homologous sequences in addition to class-specific determinants. Peptide mapping has established homologies between vimentin and desmin (21). Likewise, immunological and biochemical studies revealed extensive crossreactivity within a given class of polypeptides, keratins (22, 23). Finally, sequence studies provide

strong evidence for the existence of short regions of homology shared by wool keratin, vimentin, desmin, and tropomyosin (24).

IgM GON also detects a 66-kDal protein in various cell extracts such as fibroblast, myotube, and keratin cells and in partially purified IF. Whether this protein is constitutive of IF remains to be explored.

Similar to the findings, reported here, with the monoclonal IgM antibody produced by patient GON, the availability of hybridoma-derived monoclonal antibodies will undoubtedly uncover new crossreactivities within the family of structurally and functionally related IF proteins. Recently, a mouse hybridoma antibody has been shown to react with an antigenic determinant shared by the five classes of IF (25). Moreover, crossreaction between vimentin IF and non-IF proteins has been revealed by another monoclonal antibody (26). The existence of sequence homologies within different mammalian IF would be compatible with the concept of sequences conserved during evolution, perhaps derived from a common ancestral gene. Furthermore, such monoclonal antibodies may be valuable tools to gain insights into the functions of IF.

This study defines a new autoantibody activity for human monoclonal IgM. It is of interest that this specificity may be relatively frequent because we have found 5 of 100 WM proteins tested that show a so-called anti-smooth-muscle antibody activity. This incidence is in the same order of magnitude as that of two other autoantibody activities carried by monoclonal IgM—anti-IgG or anti–blood group Ii (27, 28). The significance of such a high incidence of some autoantibody activities among human monoclonal IgM molecules is not obvious. It has been suggested that autoantibody-synthesizing clones may preferentially undergo neoplasic transformation (29). It is conceivable that such lymphoid cells are continuously stimulated by auto- or heterocrossreactive antigens; it is therefore of note that infectious agents such as the Epstein–Barr virus or *Mycoplasma pneumoniae* may trigger the occurrence of IgM antibodies reacting with IF (30, 31).

Whether these anti-IF autoantibodies may or may not have a detrimental effect on patients remains a matter of speculation at present. It is noteworthy in this context that the anti-IF activity of the two IgMs described in this report has been detected in the course of a study of the properties of monoclonal IgM molecules produced by WM patients with peripheral neuropathy (32). The finding, in immunofluorescence experiments, of binding of these two IgMs to peripheral nerve has led to control experiments with other tissue sections whose results have suggested specificity to cytoskeletal proteins. That the monoclonal IgMs of our two patients both affected with a severe demyelinating peripheral neuropathy react with Schwann cell IF may be germane to the pathogenesis of this complication.

We thank F. Jacob, M. Seligmann, and C. Mihaesco for helpful discussions. We are grateful to P. Lemoine for photographic help and to Benoit Marin-Cudraz for technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (LA 269), the Délégation Générale à la Recherche Scientifique et Technique, the Fondation pour la Recherche Médicale Française, the Institut National de la Santé et de la Recherche Médicale (Contrat 811029), the Ligue Nationale Française contre le Cancer, the Paris VII University, and the Fondation André Meyer. K.D. is a recipient of a grant from Le Fonds d'Etude du Corps Médical.

1. Waldenström, J. (1968) *Monoclonal and Polyclonal Hypergammaglobulinemia* (University Press, London).
2. Seligmann, M. & Brouet, J. C. (1973) *Semin. Hematol.* **10,** 163–177.
3. Potter, M. (1971) *N. Engl. J. Med.* **284,** 831–838.
4. Lazarides, E. (1980) *Nature (London)* **283,** 249–256.
5. Lazarides, E. & Hubbard, B. D. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4344–4348.
6. Bignami, A., Eng, L. F., Dahl, D. & Uyeda, C. T. (1972) *Brain Res.* **43,** 429–435.
7. Lien, R. K. H., Yen, S. H., Salomon, G. D. & Shelanski, M. L. (1978) *J. Cell Biol.* **79,** 637–645.
8. Franke, W. W., Weber, K., Osborn, M., Schmid, E. & Freudenstein, C. (1978) *Exp. Cell Res.* **116,** 429–445.
9. Sun, T. T. & Green, H. (1978) *Cell* **14,** 469–476.
10. Paulin, D. (1981) *Biochimie* **63,** 347–363.
11. Franke, W. W., Schmid, E., Winter, S., Osborn, M. & Weber, K. (1979) *Exp. Cell Res.* **123,** 25–46.
12. Plaut, A. G. & Tomasi, J. B., Jr. (1970) *Proc. Natl. Acad. Sci. USA* **65,** 318–322.
13. Paulin, D., Nicolas, J. F., Yaniv, M., Jacob, F., Weber, K. & Osborn, M. (1978) *Dev. Biol.* **66,** 488–499.
14. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
15. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
16. Nicolas, J. F., Avner, P., Gaillard, J., Guenet, J. L., Jakob, H. & Jacob, F. (1976) *Cancer Res.* **36,** 4224–4231.
17. Franke, W. W., Schmid, E., Weber, K. & Osborn, M. (1979) *Exp. Cell Res.* **118,** 95–109.
18. Delacourte, A., Filliatreau, G., Boutteau, G., Biserte, G. & Schrevel, J. (1980) *Biochem. J.* **191,** 543–546.
19. Paulin, D., Babinet, C., Weber, K. & Osborn, M. (1980) *Exp. Cell Res.* **130,** 297–304.
20. Paulin, D., Robine-Léon, S. & Forest, N. (1979) *Biol. Cell.* **38,** 243–246.
21. Gard, D. L., Bell, P. B. & Lazarides, E. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3894–3898.
22. Milstone, L. M. & McGuire, J. (1981) *J. Cell Biol.* **88,** 312–316.
23. Fuchs, E. & Green, H. (1978) *Cell* **15,** 887–898.
24. Geisler, N. & Weber, K. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4120–4123.
25. Pruss, R. M., Mirsky, R., Raff, M. C., Anderton, B. & Thorpe, R. (1981) *J. Cell Biol.* **87,** 178.
26. Blose, S. H., Matsumura, F. & Lin, J. J.-C. (1981) *Cold Spring Harbor Symp. Quant. Biol.* **46,** in press.
27. Christenson, W. N., Dacie, J. V., Croucher, D. E. E. & Charlwood, P. A. (1957) *Br. J. Haematol.* **3,** 262–273.
28. Kritzman, J., Kunkel, H. G., McCarthy, J. & Mellors, R. C. (1961) *J. Lab. Clin. Med.* **57,** 905–917.
29. Metzger, H. (1969) *Am. J. Med.* **47,** 837–844.
30. Linder, E., Kurki, P. & Andersson, L. C. (1979) *Clin. Immunol. Immunopathol.* **14,** 411–417.
31. Bretherton, L., Toh, B. H. & Jack, I. (1981) *Clin. Immunol. Immunopathol.* **18,** 425–430.
32. Dellagi, K., Brouet, J. C. & Danon, F. (1979) *J. Clin. Invest.* **64,** 1530–1534.