Volume 253, number 1,2, 157–162 FEB 07502 August 1989

A new monoclonal antibody recognizing the amino-terminal consensus sequence of vertebrate intermediate filament proteins

Michel Escurat, Hai Phamgia*, Claude Huc, Annick Pouplard-Barthelaix°, Christian Boitard+, Jean-François Bach+, François Gros and Marie-Madeleine Portier

*Collège de France, Laboratoire de Biochimie Cellulaire, 75 231 Paris Cedex 05, *INSERM U 188, Hôpital Saint Vincent de Paul, Paris, °INSERM U 298, Centre Hospitalo-Universitaire, Angers* and *+INSERM U 25, Hôpital Necker, Paris, France*

Received 6 June 1989

The mouse monoclonal antibody ME 101 raised against human peripherin, an intermediate filament protein (IFP) specific to well defined neuronal populations, recognizes all the major classes of vertebrate IFP in immunoblotting assays. Desmin, GFAP, vimentin, peripherin and the lightest neurofilament protein (NF-L) were cleaved into carboxy- and amino-terminal halves by *N*-chlorosuccinimide at their unique trytophan residue. Whereas the antibody directed against the epitope common to every IFP (intermediate filament antigen or IFA) and located on the carboxy-terminal end of the rod domain recognizes the carboxy-terminal half, the ME 101 antibody, as the present study illustrates, recognizes specifically the amino-terminal half. From the amino acid sequence data of IFP, it is deduced that the cognate epitope is localized on the amino-terminal part of coil 1a.

Intermediate filament protein; Monoclonal antibody; Immunoblotting; Consensus sequence; Peripherin; Neurofilament

1. INTRODUCTION

Protein and cDNA sequence data [1–11] show that one of the main characteristics of all intermediate filament proteins (IFP), besides having two consensus sequences located in the amino-terminal part of coil 1a and at the carboxy-terminal end of coil 2 (IFA) [1,12], is the presence of a central α-helical rod domain which is well conserved in length and sequence features. In the carboxy-terminal end of coil 2, it has been shown that the common epitope is recognized by a murine monoclonal antibody elicited by Pruss et al. [13] against human glial fibrillary acidic protein (GFAP) and which allows the recognition of IFP in total protein preparations. It has been shown that this antibody is not class-specific and that it can be used to identify most IFP in both vertebrate and invertebrate tissues, thus demonstrating that there is some conservation of the rod domain in the phylogeny [13,14]. Moreover, recent data indicate that this IFA antibody also recognizes the three nuclear lamins [15], i.e. the components of the nuclear lamina, underlying the inner nuclear membrane, which have been demonstrated to possess all the major structural features of IFP [16–18].

In contrast, the existence of an antibody reacting with the amino-terminal consensus sequence of IFP has so far not been reported.

Here, we describe a mouse monoclonal antibody raised against human peripherin, an intermediate filament protein specific to well defined neuronal populations [19,20], and then show that this antibody recognizes the amino-terminal half of all the major classes of vertebrate IFP in immunoblotting assays.

Correspondence address: M. Escurat, Collège de France, Laboratoire de Biochimie Cellulaire, 75231 Paris Cedex 05, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2. MATERIALS AND METHODS

2.1. *Preparation of peripherin for immunization*

Human sympathetic ganglia, obtained through sympathectomies, were frozen in liquid nitrogen immediately after excision. Homogenates, prepared as already described [19], were immediately treated for electrofocusing. Proteins were separated by two-dimensional gel electrophoresis [21] and stained with Coomassie blue. The spot corresponding to peripherin was cut out from 50 gels; in order to remove the stain and to concentrate the protein, the excised spots were all loaded on a 12.5% polyacrylamide slab gel; after electrophoresis, 2 cm-wide strips were cut out from lateral edges of the gels, stained for 15 min with Coomassie blue and aligned back into position on the unstained gel; this permitted the localization of the peripherin band on the unstained portion of the gel. The band was excised, washed with phosphate buffered saline (PBS) for 2 h by changing buffer four times, homogenized in a glass-Teflon Potter homogenizer. Sterile PBS was then added until the slurry was fluid enough to be pumped through a syringe.

2.2. *Immunization and production of hybridomas*

Balb/c mice were immunized by injecting into the foot pads the gel-slurry emulsified with an equal volume of complete Freund's adjuvant for the first shot, and with incomplete Freund's adjuvant for the subsequent shots on days 7, 14 and 24. Three days after the last injection, spleen cells were isolated and used for somatic cell hybridization with the myeloma cell line P3 × 63 Ag 865.3 [22] according to the slightly modified [24–26] method of Kohler and Milstein [23]. Hybridomas were distributed in 96 microwell culture plates and grown in HAT medium (RPMI containing 15% fetal calf serum, 1% sodium pyruvate, 1% essential amino acids, 1% penistreptoglutamine).

2.3. *Selection of hybridomas*

Supernatants from wells containing proliferating hybridomas were assayed by immunoblotting. Total cellular extracts from human sympathetic ganglia were loaded on whole width of single 12.5% polyacrylamide slab gels, electrophoresed for 4–5 h and the proteins were then transferred to a nitrocellulose membrane in a Transfor apparatus; after saturation with 3% bovine serum albumin and washes as described by Towbin et al. [27], the membrane was cut to the right size and positioned in a 28-lane miniblotter apparatus (Immunetics, Cambridge, MA, USA). Each supernatant was introduced into a single lane. Using two such devices, 50 supernatants could be tested each day and the remaining lanes being used for the controls. We chose as a standard, the IFA antibody elicited by Pruss et al. [13]. The supernatants which reacted with proteins migrating at positions between 50 and 60 kDa were then cross-checked with the proteins separated on two-dimensional polyacrylamide gels and transferred onto nitrocellulose membranes. Hybridomas reacting either with peripherin or with every IFP were selected. Supernatants from the subclones were tested identically. Selected hybridomas were expanded and obtained in the form of ascites after intraperitoneal injection into Swiss nude mice.

The immunoglobulin subclass of the antibody was determined by Mancini double immunodiffusion analysis using rabbit anti-mouse immunoglobulin in quantitative immunodiffusion plates (Meloy Laboratories Inc., Springfield, VA).

2.4. *Cell and tissue extracts*

Lysates from the mouse neuroblastoma NIE 115 cell line, and homogenates of human brain and sympathetic ganglia, of mouse and rabbit spinal cord and sciatic nerve, and of chicken gizzard and sciatic nerve were prepared as already described [19]; they were then treated for isoelectric focusing (IEF) [21] and stored at −30°C. Cytokeratins from oral epithelia were extracted as described by Franke et al. [28] and treated for non-equilibrium pH gradient electrophoresis (NEPHGE) [29]; they were numbered according to Moll et al. [30].

2.5. *SDS-polyacrylamide gel electrophoresis*

Proteins were separated on two-dimensional polyacrylamide gels [21] and were either transferred immediately onto nitrocellulose membranes or stained with Coomassie blue and excised for peptide analyses. All these methods have already been described [19].

2.6. N-*Chlorosuccinimide treatment*

Protein spots cut out from two-dimensional gels were treated with *N*-chlorosuccinimide as described [31]. Peptides were resolved and analyzed on 15% slab gels containing 0.1% SDS.

2.7. *Immunoblotting*

Proteins separated by polyacrylamide gel electrophoresis were transferred onto nitrocellulose membranes [27] in a Transfor apparatus at 500 mA for the 8% acrylamide two-dimensional gels and at 700 mA for the 15% acrylamide slab gels; in both cases the transfer time was 1 h. Proteins and peptides were visualized on the membranes by staining with Ponceau red as described [19], and their positions were marked as points with a pencil.

Immunostaining was as described by Dellagi et al. [32]. The hybridoma ascites fluid was used at a dilution of 1:100. The murine IFA hybridoma clone was a gift from Dr B.H. Anderton (St. George's Hospital, London); it was used as a hybridoma supernatant diluted 1:1.

3. RESULTS

3.1. *Selection of hybridomas*

Firstly, hybridomas were selected on miniblots onto which proteins from total extracts of either human sympathetic ganglia or mouse NIE 115 neuroblastoma cells had been transferred after separation on slab gels (not shown). This enabled one to check a number of hybridomas simultaneously under identical conditions and against total cell extracts containing not only the protein under study but also many other similar proteins. For example, a total extract from human sympathetic ganglia contains several IFP (the triplet of neurofilament proteins, vimentin and peripherin), whereas that from mouse neuroblastoma contains the co-expressed vimentin and peripherin. Despite the fact that the molecular

masses of the latter two proteins are almost identical, they are only clearly separated on two-dimensional gels. This is why the hybridomas were first selected on miniblots and then checked against total cell or tissue proteins separated on two-dimensional gels.

The results obtained with hybridoma ME 101, whose antibody belongs to the IgM class are shown in fig.1. Clearly, it reacts with the lightest neurofilament protein (NF-L), vimentin and its derived peptides [33], the different isoforms of peripherin [34], GFAP, desmin and every cytokeratin present in the preparation from human oral epithelia. These data initially led us to postulate that ME 101 behaves like the previously described IFA antibody elicited by Pruss et al. [13]. This reagent recognizes the consensus sequence common to every IFP which is located at the carboxy-terminal end of the rod domain. However, the subsequent experiments clearly

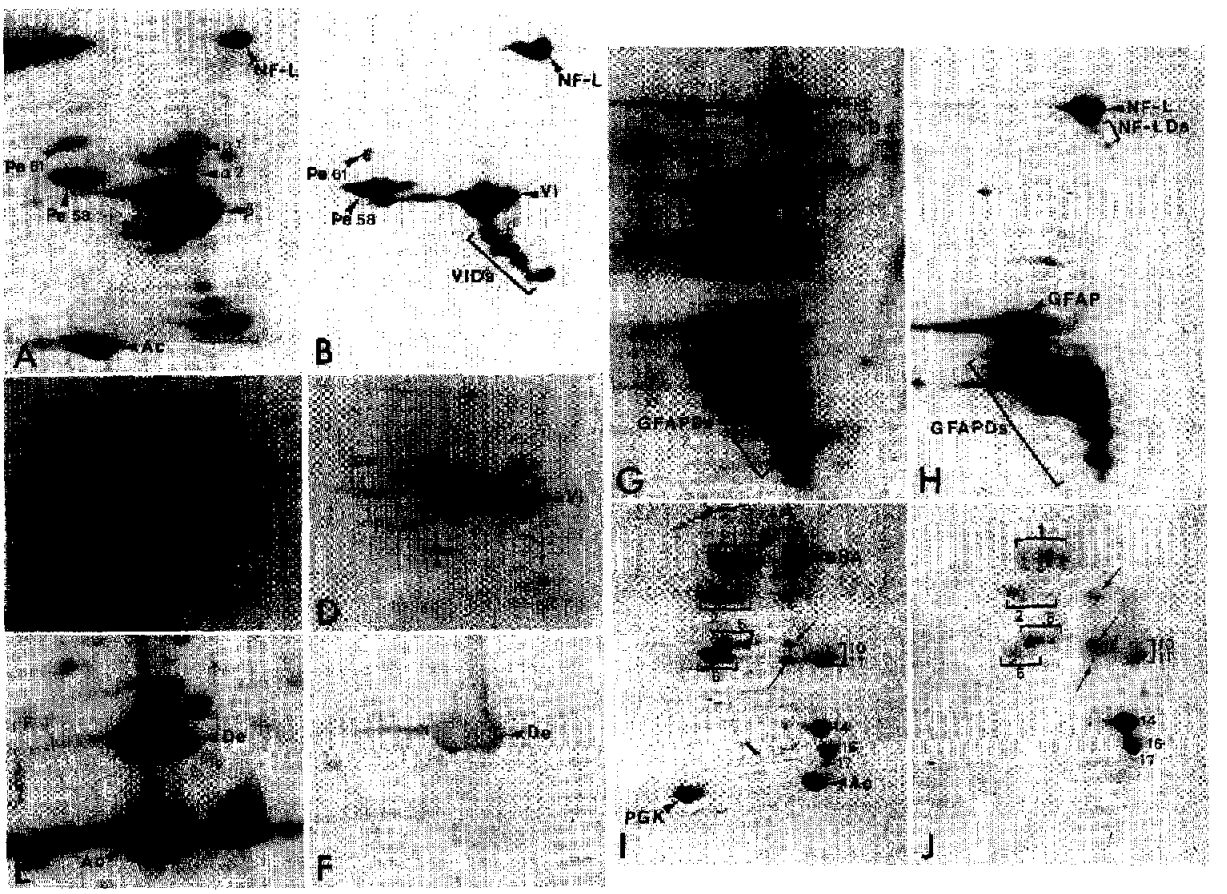

Fig.1. Immunoreactivity of intermediate filament proteins to ME 101. (A) Portion of a Coomassie blue stained two-dimensional gel (8% acrylamide–0.09% bisacrylamide) loaded with an extract from human sympathetic ganglia. (B) Western blot of A and immunodetection with ME 101. (C) As in A but the extract was from mouse neuroblastoma NIE 115 cell line. (D) Western blot of C and as in B. (E) As in A and C but the extract was from chicken gizzard. (F) Western blot of E and as in B. (G) As in A, C and E but extract was from human mesencephalon. (H) Western blot of G and as in B. (I) Portion of a Ponceau red stained blot. The first-dimensional gel (NEPHGE) had been loaded with a preparation of cytokeratins from human oral epithelia. The concentration of acrylamide in the second-dimensional gel was 10% with 0.27% bisacrylamide. (J) Immunodetection of I and as in B. Pe 61, Pe 58, Pe 56, isoforms of peripherin; Vi, vimentin; ViDs, vimentin-derived proteins; NF-L, the lightest neurofilament protein; NF-L Ds, NF-L-derived proteins; GFAP, glial fibrillary acidic protein; GFAP Ds, GFAP-derived proteins; De, desmin; 1,2,5..., cytokeratins; arrows, complexes formed between type I and type II keratins; α_1, α_2, β, tubulin subunits; Ac, actin; PGK, phosphoglucokinase; BSA, bovine serum albumin. These three last proteins were added as markers in I.

established that our monoclonal antibody displays a different specificity. It is remarkable that the intensity of the reaction differs according to the IFP and the antibody used. This is particularly evident when the cell extract loaded onto the gel contains several different IFP. For instance, in extracts from human sympathetic ganglia, peripherin is first decorated to a very large extent by the IFA antibody, then vimentin and lastly, NF-L. In contrast, using ME 101, NF-L is first decorated very intensely, followed by vimentin and peripherin. In extracts from human brain, NF-L was also decorated very strongly by the ME 101 antibody but only weakly by the IFA antibody. No significant reaction could be detected with the two heaviest NFP subunits on two-dimensional polyacrylamide gels, using either antibody; a reaction was, however, observed with the IFA antibody when purified NFP were separated on slab gels [14]. One may ask whether the largest NF subunits undergo complete denaturation during two-dimensional gel electrophoresis in view of the fact that the first dimension gels are only equilibrated in a SDS-containing buffer whereas in the case of slab gels the protein samples are boiled with SDS.

3.2. *Recognition of the carboxy-terminal moiety of IFP*

Geisler et al. [35] have demonstrated that desmin, NF-L, vimentin and GFAP contain a unique tryptophan residue sited in the central part of the rod domain. We have demonstrated that peripherin also displays a single tryptophan residue at this same location [19]. Combination of the *N*-chlorosuccinimide treatment, which cuts the molecules specifically at their tryptophan residue, with immunostaining of the peptides, transferred onto a nitrocellulose membrane after separation on a slab gel, using the IFA antibody allows one to localize the carboxy-terminal moiety of IFP as shown in fig.2. This reactivity is found to reside in the lightest peptide for peripherin, vimentin and GFAP, and in the heaviest one for NF-L; this is in agreement with available data concerning their respective molecular masses which have been shown to depend mostly on the length of the carboxy-terminal tail domain [12].

3.3. *Localization of the common epitope recognized by ME 101*

The same experiment was repeated applied to ME 101 with IFP from several origins (human

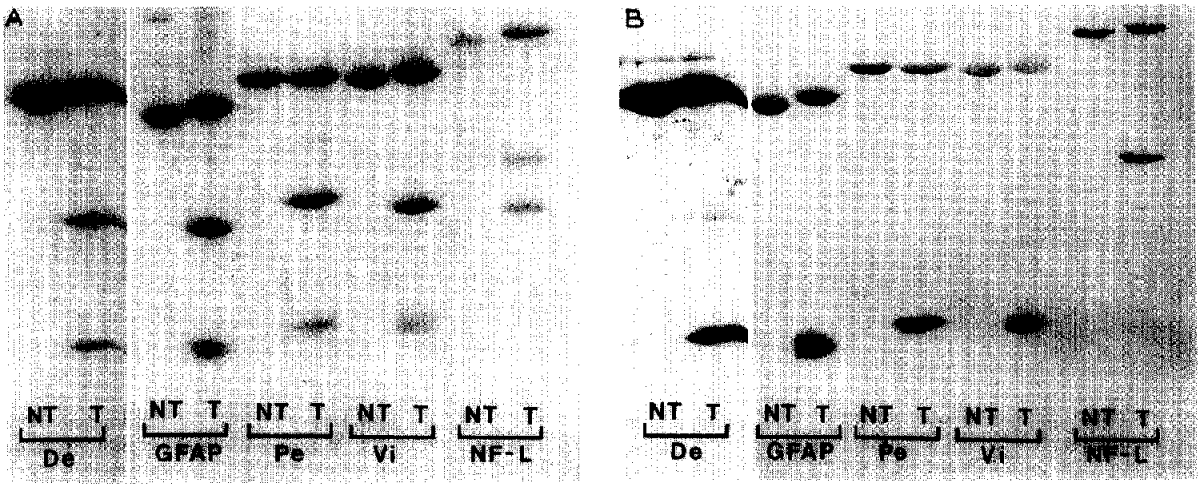

Fig.2. Determination of the carboxy-terminal moiety of some intermediate filament proteins. (A) Coomassie blue staining of the peptides cleaved from intermediate filament proteins with *N*-chlorosuccinimide. Peptides were separated on gels containing 15% acrylamide and 0.2% bisacrylamide. (B) Immunodetection of the carboxy-terminal moiety by decoration with the IFA antibody. The gels shown in A were blotted partly before Coomassie blue staining so that Coomassie blue and Ponceau red stainings coincided perfectly. After Ponceau red staining, the bands were marked on the blot as points with a pencil. Immunodetection of the carboxy-terminal moiety was carried out with the IFA antibody. T, *N*-chlorosuccinimide treated protein; NT, non-treated protein. Other abbreviations are as in fig.1.

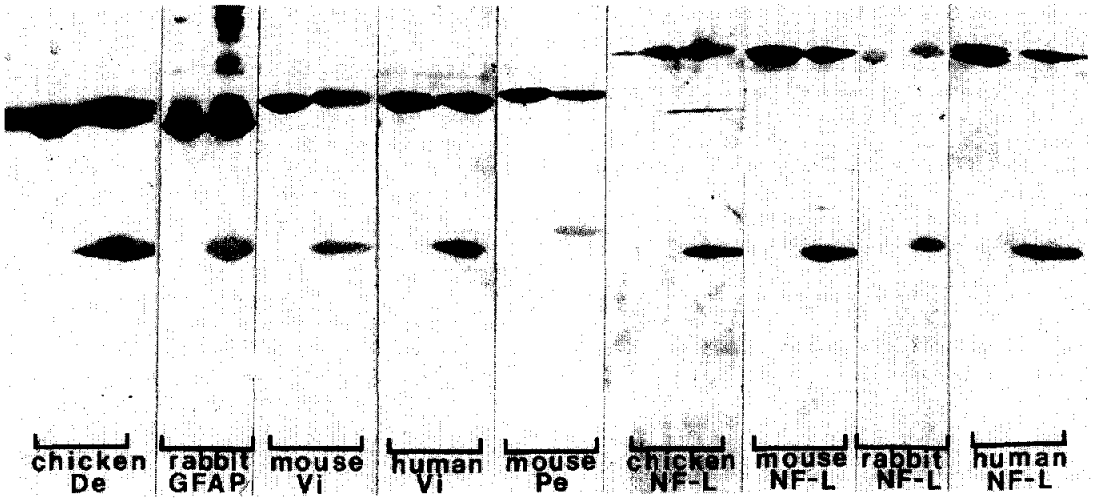

Fig.3. Immunodetection of the amino-terminal moiety with ME 101. Peptides were prepared, separated and blotted as in fig.2. Immunodetection was carried out with ME 101. Abbreviations are as in fig.2.

brain and sympathetic ganglia, mouse neuroblastoma NIE 115 cell line, rat sciatic nerve, rabbit sciatic nerve and spinal cord, chicken gizzard and sciatic nerve). From the results shown in fig.3, it is clear that ME 101 recognizes the amino-terminal moiety of NF-L, peripherin, vimentin, desmin and GFAP.

4. DISCUSSION

ME 101 recognizes every IFP so far tested after transfer onto nitrocellulose membranes and it is clear from our results that the common epitope thus recognized is located on the amino-terminal half of the IFP. From the sequence data relative to these molecules [1–11], it can be inferred that ME 101 recognizes the consensus sequence located on coil 1a. Such an antibody may represent, as does the IFA antibody, an interesting tool for detecting IFP and their degradation products in cell extracts; it may be particularly useful in recognizing the rod domain or its amino-terminal half among the products resulting from specific enzymatic or chemical degradations. However, since it has been demonstrated that the IFA antibody can also recognize the lamins [15], it would be of interest to determine if it is also the case with ME 101. From the sequence data [16,17], it seems that lamins also possess the consensus sequence located on coil 1a of the rod domain, demonstrating the highly conserved nature of this portion of the molecule in the IFP.

The IFA antibody has also been shown to exhibit a broad cross-species reactivity, since it recognizes IFP from many invertebrates [13,14]. It will be interesting to determine whether this would also be the case for ME 101 which would then constitute an additional marker for such studies; this would bring a supplementary element to the demonstration of the conservation of the rod domain during the phylogenetic evolution of IFP.

Acknowledgements: The authors thank Dr C. Sripati for helpful suggestions and Dr F. Gosselin for a gift of keratins. This work was supported by a grant from the Ministère de la Recherche et de l'Enseignement Supérieur (86C 0964).

REFERENCES

[1] Geisler, N. and Weber, K. (1982) EMBO J. 1, 1649–1656.
[2] Quax, W., Van den Broek, L., Egberts, W.V., Ramaekders, F. and Bloemendal, H. (1985) Cell 43, 327–338.
[3] Geisler, N. and Weber, K. (1983) EMBO J. 2, 2059–2063.
[4] Quax, W., Egberts, W.V., Hendriks, W., Quax-Jeuken, Y. and Bloemendal, H. (1983) Cell 35, 215–223.
[5] Geisler, N., Plessman, U. and Weber, K. (1985) FEBS Lett. 82, 475–478.
[6] Lewis, S.A. and Cowan, N.J. (1986) Mol. Cell. Biol. 6, 1529–1534.

[7] Lewis, S.A., Balcarek, J.M., Krek, V., Shelanski, M. and Cowan, N.J. (1984) Proc. Natl. Acad. Sci. USA 81, 2743–2746.
[8] Geisler, N., Fischer, S., Vandekerckhove, J., Plessmann, U. and Weber, K. (1984) EMBO J. 3, 2701–2706.
[9] Myers, M.W., Lazzarini, R.A., Lee, V.M.-Y., Schlaepfer, W.W. and Nelson, D.L. (1987) EMBO J. 6, 1617–1626.
[10] Geisler, N., Fischer, S., Vandekerckhove, J., Van Damme, J., Plessmann, U. and Weber, K. (1985) EMBO J. 4, 57–63.
[11] Robinson, P.A., Wion, D. and Anderton, B.H. (1986) FEBS Lett. 209, 203–205.
[12] Geisler, N., Kaufmann, E., Fischer, S., Plessmann, U. and Weber, K. (1983) EMBO J. 2, 1295–1302.
[13] Pruss, R.M., Mirsky, R., Raff, M.C., Thorpe, R., Dowding, A.J. and Anderton, B.H. (1981) Cell 27, 419–428.
[14] Pruss, R.M. (1985) J. Neuroimmunol. 8, 293–299.
[15] Osborn, M. and Weber, W. (1987) Exp. Cell Res. 170, 195–203.
[16] McKeon, F.D., Kirschner, M.W. and Caput, D. (1986) Nature 319, 463–468.
[17] Fischer, D.Z., Chaudhary, N. and Blobel, G. (1986) Proc. Natl. Acad. Sci. USA 83, 6450–6454.
[18] Aebi, U., Cohn, J., Buhle, L. and Gerace, L. (1986) Nature 323, 560–564.
[19] Portier, M.-M., De Néchaud, B. and Gros, F. (1984) Dev. Neurosci. 6, 335–344.
[20] Escurat, M., Gumpel, M., Lachapelle, F., Gros, F. and Portier, M.-M. (1988) CR Acad. Sci. Paris 306, 447–456.
[21] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[22] Galfre, G., Howe, S.C., Milstein, C., Butcher, G.W. and Howard, J.C. (1977) Nature 266, 550–552.
[23] Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.
[24] Oi, V.T., Jones, P.P., Goding, J.W., Herzenberg, L.A. and Herzenberg, L.A. (1978) Curr. Top. Microbiol. Immunol. 81, 115–129.
[25] Oi, V.T. and Herzenberg, L.A. (1980) in: Selected Methods in Cellular Immunology (Mishell, B.B. and Shiigi, S.M. eds) pp.351–372, W.H. Freeman and Co., San Francisco.
[26] Kearney, J.F., Radbruch, A., Liesegang, B. and Rajewsky, K. (1979) J. Immunol. 123, 1548–1550.
[27] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[28] Franke, W.W., Schiller, D.L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R. and Platzer, B. (1981) J. Mol. Biol. 153, 933–959.
[29] O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell 12, 1133–1142.
[30] Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. (1982) Cell 31, 11–24.
[31] Lischwe, M.A. and Ochs, D. (1982) Anal. Biochem. 127, 453–457.
[32] Dellagi, K., Brouet, J.C., Perreau, J. and Paulin, D. (1982) Proc. Natl. Acad. Sci. USA 79, 446–450.
[33] Ochs, D.C., McConkey, E.H. and Guard, N.L. (1981) Exp. Cell. Res. 135, 355–362.
[34] Landon, F., Lemonnier, M., Benarous, R., Huc, C., Fiszman, M., Gros, F. and Portier, M.M. (1989) EMBO J., in press.
[35] Geisler, N., Plessmann, U. and Weber, K. (1982) Nature 296, 448–450.