Thymomatous Epithelial Cells and Skeletal Muscle Share a Common Epitope Defined by a Monoclonal Antibody

MIREILLE DARDENNE, MD,
WILSON SAVINO, MD, PhD, and
JEAN-FRANÇOIS BACH, PhD

From Hôpital Necker, Paris, France

Monoclonal antibodies have been raised against thymomatous epithelial cells with the use of fragments of a human thymoma as source of antigen. These monoclonals, which do not react with cultured epithelial cells or sections from normal thymus (except for some cells of Hassall's corpuscles), label a large number of cells in thymoma sections. In addition, they recognize cross-striations from skeletal muscle cells. Immunoblotting studies reveal that the proteins recognized on thymoma and muscle are in the same molecular weight range, suggesting that these proteins, which share a common epitope, are identical. These findings indicate that the production of circulating anti-cross-striational autoantibodies observed in most patients with thymoma could derive from immunization against a protein abnormally expressed by neoplastic epithelial cells. (Am J Pathol 1987, 126:194–198)

THYMOMAS are defined as neoplastic proliferations of thymic epithelial cells (TECs)[1] around which one usually finds variable amounts of lymphocytes, predominantly those bearing the T6 phenotype, normally expressed by cortical thymocytes.[2] In a series of studies recently performed in our laboratory, we demonstrated that these neoplastic cells present several alterations in their expression of cytoplasmic and membrane proteins. Thus, we observed that thymomatous epithelial cells secrete large amounts of thymulin (a chemically defined thymic hormone). Additionally, they usually do not express Class II antigens of the major histocompatibility complex,[3] present on normal TECs[4,5] and express abnormally low amounts of cytoskeletal differentiation antigen.[6] In contrast, the carcinoembryonic antigen, which is not borne by normal TECs, is detected on these neoplastic cells.[7] Paralleling these phenotypic changes is the common occurrence of autoantibodies, notably against acetylcholine receptor (AChR),[8] nuclear antigens,[9] and especially the so-called anti-cross-striational antibodies[10–11] that recognize cytoplasmic proteins at the level of the cross-striation in skeletal muscle cells. The mechanisms leading to the activation of these anti-skeletal muscle autoantibody-secreting cells remain unknown. The demonstration of AChR-like proteins on human TECs[12,13] and the occurrence of anti-AChR antibody-synthesizing cells within the thymus[14] suggest the hypothesis of a primary intrathymic sensitization as far as the anti-AChR antibodies are concerned. No such direct data had so far been reported for anti-striational antibodies. However, that sera from thymoma patients can be shown (using an immunofluorescence assay) to react with thymomatous epithelial cells and skeletal muscle[11] and that antisera produced against skeletal muscle extracts bind to thymomatous cells[15] suggest the existence in these two tissues of common epitopes, probably distinct from AChR. These observations prompted us to produce monoclonal antibodies by immunizing mice with fragments of a human thymoma. The study of such monoclonal antibodies (MAb's) provided evidence for the existence of epitopes shared by thymomatous epithelial cells and normal skeletal muscle cells.

Accepted for publication August 27, 1986.

Address reprint requests to M. Dardenne, INSERM U 25, Hôpital Necker, 161, rue de Sèvres, 75730 PARIS Cedex 15, France.

Materials and Methods

Reagents

The chemicals used, including those used for hybridoma production, gel electrophoresis, and Western blotting, were purchased from Sigma Chemical Company (St. Louis, Mo) unless specified otherwise. Dulbecco's modified Eagle's medium and RPMI 1640 were obtained from GIBCO (Paisley, Scotland), and fetal calf serum was purchased from Flow Laboratories (Puteaux, France). Acrylamide and bisacrylamide were Serva products (Heidelberg, Germany), and nitrocellulose sheets were bought from Cera Laboratories (Aubervilliers, France).

The anti-keratin xenoantiserum (Clinisciences, Paris) was raised in rabbits after immunization with purified human epidermal keratins.[16] Previous studies in our laboratory demonstrated that this antiserum (here used diluted 1 : 50) strongly and specifically labels the epithelial network of both normal and hyperplastic human thymuses as well as thymomas.[3] The fluorescent conjugates used as second antibodies in immunohistochemical analyses were purchased from Nordic Laboratories, Tilburg, The Netherlands. They were absorbed with acetone liver powder (Sigma) so that nonspecific fluorescent labeling could be avoided. The GAR/TRITC (goat anti-rabbit immunoglobulins bound to tetramethyl rhodamine isothiocyanate) as well as the GAM/FITC (goat anti-mouse immunoglobulins coupled to fluorescein isothiocyanate) were used at a dilution of 1 : 50. For immunoblot studies we used a GAM/Fab'/PO (GAM Fab' fragment bound to peroxidase) obtained from Amersham (Buckinghamshire, England). This conjugate was used at a dilution of 1 : 500.

Thymus Samples

Normal human thymus fragments were kindly provided by Dr. J. Y. Neveux (Hôpital Laennec, Paris). These fragments were obtained from 12 children (aged 6 months to 2 years) undergoing cardiac surgery. In addition, we studied five hyperplastic thymuses from myasthenia gravis (MG) patients and six thymomas (associated or not with MG) provided by Sonia Berrih (Centre Chirurgical Marie Lannelongue, Plessis-Robinson, France). The thymoma sample used for immunizing mice was obtained from a patient with no signs of MG but exhibiting high levels of circulating anti-AChR antibodies. Immediately after surgical removal, all fragments except those used for establishing primary TEC culture were frozen in liquid nitrogen and stored at −173 C.

Other Organs

The monoclonal antibodies were tested by immunofluorescence on frozen sections of several other organs (obtained at autopsies performed at our hospital), namely, skin, esophagus, stomach, urinary bladder, liver, lung, heart, spleen, and skeletal muscle.

Immunization and Cell Fusion

Six-week-old BALB/c mice were given small fragments of the thymoma. Intraperitoneal booster injections were carried out at 2–3-week intervals. Four days after the third booster immunization, the mice were killed and cell fusion with PEG was performed according to the procedure routinely used in our laboratory.[17] The cultures were maintained for 14 days in hypoxanthine-aminopterine-thymidine (HAT) medium, which was then replaced by hypoxanthine-thymidine (HT) medium. Supernatants from the wells containing hybridomas were screened by immunofluorescence for their capacity to bind to frozen sections of the thymoma used for immunization. Cells from positive wells were then cloned and subcloned by limiting dilutions and subsequently grown as tumor ascites in BALB/c mice primed with Pristane.

Thymic Epithelial Cell Cultures

Primary cultures of human thymic epithelium were established according to the technique previously reported.[18] Briefly, fresh thymus samples were minced in RPMI 1640, and fragments were allowed to settle for 10 minutes in 6-well culture dishes. The culture medium (RPMI 1640 supplemented with 20% fetal calf serum, 1% L-glutamine, and 2% penicillin-streptomycin) was changed twice a week. Proliferating large polyclonal cells were observed by Day 3, and confluence was attained by Days 12–14. The epithelial nature of the cells in culture was assessed by the presence of tonofilaments and desmosomes[18] and by their keratin content.[19]

Immunofluorescence Technique

Unfixed, as well as acetone-fixed, 2-mm-thick frozen thymic sections were processed as described below. At the screening stage, the sections were incubated with undiluted supernatants from hybrid (or clone) cultures for 30 minutes, washed in phosphate-buffered saline (PBS) for 15 minutes, and finally incubated with the GAM/FITC for 30 minutes. After a second wash with PBS, the sections were mounted under coverslips with glycerol/PBS. Ascitic fluids

(0.01 mg Ig/ml) obtained from mice given injections of positive clones were similarly studied. An unrelated ascites with the same Ig concentration was systematically used as a control.

In double-labeling experiments, the anti-thymoma ascites was revealed by the GAM/FITC and the anti-keratin serum was visualized with the GAR-TRITC. Four additional controls were used in such a way, so that in each case, one of the specific antibodies was missing. These controls confirmed the absence of nonspecific labeling.

Gel Electrophoresis and Immunoblot Analysis

Extracts (containing 2-β-mercaptoethanol) from thymoma, normal thymus, or skeletal muscle were subjected to sodium dodecyl sulfate containing polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli's technique,[20] with 10% acrylamide homogeneous 1.5-mm-thick gels and Tris-glycine as the running buffer.

For immunoblot analysis, the protein bands resolved within the gels were transferred onto nitocellulose (NC) sheets as initially described by Towbin et al.[21] The NC sheets were stained with 0.2% Ponceau red (in 3% trichloroacetic acid) to check the transfer and were then saturated for 1 hour with 3% bovine serum albumin (BSA) diluted in PBS. After washing with 0.5% BSA, 0.2% Tween 20 in PBS, they were incubated with the anti-thymoma monoclonal antibody for 18 hours at room temperature, washed in BSA/Tween/PBS, and exposed to the GAM/Fab'/PO for 2 hours. After another washing, peroxidase activity was revealed with 0.03% H_2O_2 in the presence of 0.5 mg/ml 3, 3'-diaminobenzidine in 0.1 M Tris buffer, pH 7.6.

Results

Four positive hybrids producing antibodies binding to thymoma sections were detected in three fusion experiments. The supernatant showing the most intense fluorescence was selected for further studies. This antibody was shown by immunodiffusion to be of the IgG2 isotype.

Thymic Reactivity of the Anti-Thymoma MAb

When sections of normal or hyperplastic (HG) human thymuses were incubated with the anti thymoma MAb in the immunofluorescence assay, the epithelial reticulum remained virtually negative (Figure 1) except for some cells of Hassall's corpuscles and rare isolated cells near these structures. Primary cultures of normal human TECs did not exhibit any cells reacting with the antibody. This result was obtained both on unfixed and acetone-fixed cultures.

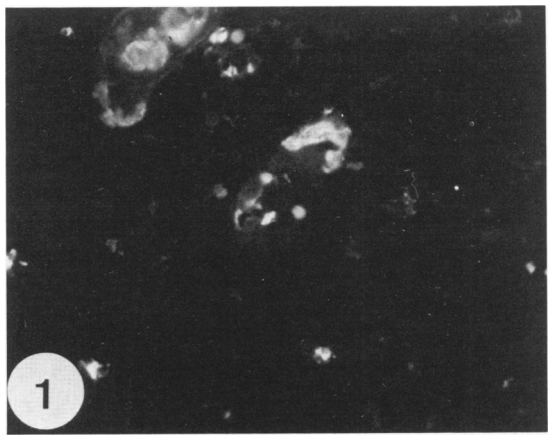

Figure 1—Immunofluorescent labeling of a normal human thymus section, using the antithymoma MAb (revealed with the GAM/FITC). Thymic epithelial reticulum is virtually negative except for some cells of Hassall's corpuscles. (×450)

In contrast to such data on the normal thymus, a large number of labeled cells was consistently observed to react with the MAb in thymoma sections (Figure 2). The pattern of labeling revealed a cellular network typical of neoplastic epithelial cells, a finding further supported by the keratin positivity of these cells ascertained in double-labeling experiments (data not shown).

Extrathymic Binding of the Anti-Thymoma MAb

The anti-thymoma MAb showed a significant binding to frozen sections of skeletal muscle. The

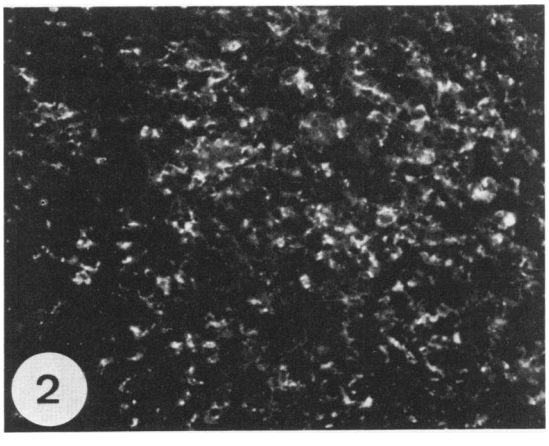

Figure 2—Frozen section of a thymoma immunolabeled by the anti-thymoma MAb. A typical fluorescent cellular reticulum is shown in this field. The epithelial nature of the labeled cells was confirmed by their keratin positivity (data not shown). (×450)

binding was essentially cytoplasmic at cross-striations of skeletal muscle cells (Figure 3). Importantly, all other organs tested, including heart, did not exhibit any reactivity with the anti-thymoma MAb.

Immunochemical Identification of the Proteins Reacting With the Anti-Thymoma MAb

When thymomatous extracts (previously resolved by SDS-PAGE and transferred onto NC sheets) were allowed to react with the antithymoma MAb, two major bands were detected. They had molecular weights (MW) of 109 and 112 kd. They were not found in resolved normal thymus extracts except for a slight reaction, in the same molecular weight range, when the MAb was used five times more concentrated. In contrast, two bands having similar molecular weights were clearly identified in the lanes containing skeletal muscle extracts (Figure 4).

Discussion

The present study shows that neoplastic thymic epithelial cells express a protein virtually absent from the normal human thymic epithelial network but shared by skeletal muscle cells. In this respect, the anti-thymoma MAb-defined protein might be regarded as a dedifferentiation marker of human TECs. The rare observation of MAb cells reactive in Hassall's corpuscles in normal thymus remains unclear, but the slight labeling (in the same molecular weight range) observed in the immunoblot studies when the MAb was applied more concentrated indicates that these structures express the same immunoreactive proteins detected in thymomatous extracts. It is interesting to note that another dedifferentation protein, namely,

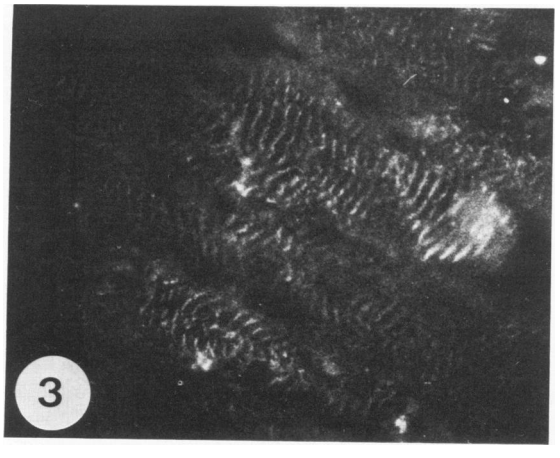

Figure 3 — Immunofluorescent staining on a frozen section of human skeletal muscle incubated with the anti-thymoma MAb. The labeling here observed is clearly at the level of muscle cell cross-striations. (×600)

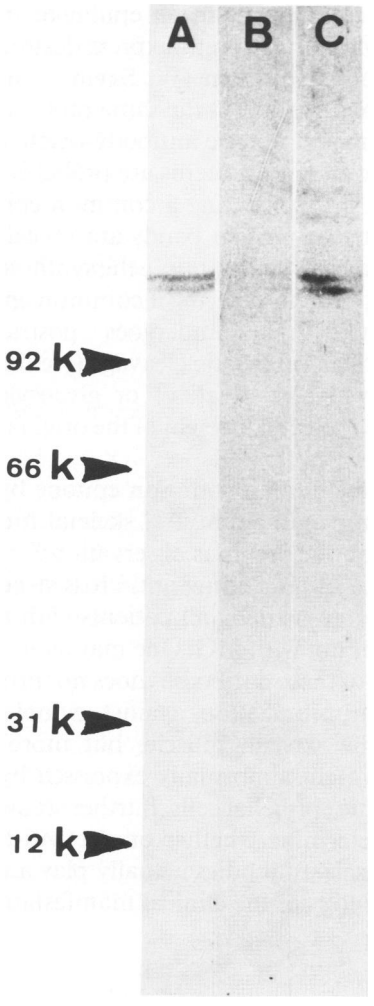

Figure 4 — Immunoblot analysis of thymoma (lane A), normal thymus (lane B), or skeletal muscle (lane C) extracts after labeling with the anti-thymoma MAb. In lanes A and C ascitic fluid was applied diluted 1 : 100 (corresponding to 0.01 mg Ig/ml), whereas in lane B it was diluted 1 : 10. Two major bands of 109–112 kd are clearly seen in both thymoma and skeletal muscle extract (A and C). A slight labeling (in the same molecular weight range) can be detected in the normal human thymus extract only when the anti-thymoma MAb is applied concentrated five times more (lane B).

the carcinoembryonic antigen (a 180–200-kd protein) is also strongly expressed by neoplastic and cultured human TECs, but not in the normal organ, except for some cells in Hassall's corpuscles.[7]

The epitope to which the anti-thymoma MAb binds is also present in the cross-striations of skeletal muscle cells, as shown both by immunofluorescence and immunoblotting studies. This finding raises the question whether the cells recognized in the normal thymus and/or thymomas might be the so-called myoid cells, seldom observed in normal conditions[22] and detected in some thymomas.[23] However, this has not proven to be the case, because anti-thymoma MAb-binding cells were consistently keratin-positive

(thus characterizing them as epithelial in nature), whereas thymic myoid cells express desmin as intermediate filament protein (W. Savino, unpublished data). The observation of the same molecular weight for thymoma and muscle antibody-reactive proteins suggests that the two proteins are probably identical, rather than simply sharing a common epitope. The finding that two protein bands are revealed by the anti-thymoma MAb may imply the synthesis of either two distinct proteins sharing a common epitope or a single protein that undergoes postranslational changes such as enzymatic cleavage (yielding a lower molecular weight derivative) or glycosylation (increasing the molecular weight of the original polypeptide).

The expression of a common epitope by the neoplastic thymic epithelium and skeletal muscle casts some light on the previous observation[10,11] regarding the existence of circulating anti-cross-striational autoantibodies in virtually all patients with thymoma, associated or not with MG. One may assume that the production of these antibodies does not primarily derive from immunization against cytoplasmic elements of the skeletal muscle, but more probably against a protein abnormally expressed by the neoplastic thymic epithelial cells. Further studies are necessary to determine whether or not anti-thymoma/skeletal muscle antibodies actually play a role in the pathophysiology of the clinical manifestation of thymomas.

References

1. Levine GD, Rosai J: Thymic hyperplasia and neoplasia: a review of concepts. Hum Pathol 1978, 9:495–515
2. Kornstein MJ, Hoxie JA, Levinson AI, Brooks JJ: Immunohistology of thymomas. Arch Pathol Lab Med 1985, 109:460–463
3. Savino W, Manganella G, Verley JM, Wolff A, Berrih S, Levasseur P, Binet JP, Dardenne M, Bach JF: Thymoma epithelial cells secrete thymic hormone but do not express class II antigens of the major histocompatibility complex. J Clin Invest 1985, 76:1140–1146
4. Janossy GJ, Thomas A, Bollum FJ, Granger S, Pizzolo G, Bradstock KF, Wong L, Mac Michael A, Ganeshaguru K, Bradstock AV: The human thymic microenvironment: an immunohistologic study. J Immunol 1980, 125:202–212
5. Rouse RV, Parham P, Grumet FC, Weissman IL: Expression of HLA antigens by human thymic epithelial cells. Hum Immunol 1982, 5:21–34
6. Savino W, Berrih S, Dardenne M: Thymic epithelial antigen acquired during ontogeny and defined by the anti p19 monoclonal antibody is lost in thymomas. Lab Invest 1984, 51:292–296
7. Savino W, Durand D, Dardenne M: Immunohistochemical evidence for the expression of the carcinoembryonic antigen by human thymic epithelial cells in vitro and in neoplastic conditions. Am J Pathol 1986, 121:418–425
8. Lefvert AK, Gergstrom A, Matell G, Osterman PO, Pirskanen R: Anti-acetylcholine receptor antibodies in myasthenia gravis: Part 1. Relation to clinical parameters in 250 patients. J Neurol Sci 1983, 58:357–370
9. Warlow R, Garlepp M, Dawkins R: Extractable nuclear antigen autoantibodies and their association with other autoantibodies and thymoma in myasthenia gravis. J Neuroimmunol 1985, 8:185–197
10. Oosterhuis HJ, Feltkamp JE, Van Rossum AL, Vand der Berg-Loonen PM, Mijenhuis LE: HLA antigens, autoantibody production and associated diseases in thymoma patients, with or without myasthenia gravis. Ann NY Acad Sci 1976, 274:468–474
11. Aarli JA, Lefvert AK, Tonder O: Thymoma-specific antibodies in sera from patients with myasthenia gravis demonstrated by indirect haemagglutination. J Neuroimmunol 1981, 1:421–427
12. Engel WK, Trotter JL, McFarlin DE, McIntosh CL: Thymic epithelial cell contains acetylcholine receptor. Lancet 1981, 1:1310–1311
13. Raimond F, Morel E, Bach JF: Evidence for the presence of immunoreactive acetylcholine receptors on human thymus cells. J Neuroimmunol 1984, 6:31–40
14. Newson-Davis J, Willcox N, Calder L: Thymus cells in myasthenia gravis selectively enhance production of anti-acetylcholine-receptor antibody by autologous blood lymphocytes. N Engl J Med 1981, 305:1313–1318
15. Gilhus NE, Aarli JA, Christensson B, Matre R: Rabbit antiserum to a citric extract of human skeletal muscle staining thymomas from myasthenia gravis patients. J Neuroimmunol 1984, 7:55–64
16. Viac J, Staquet MJ, Thivolet J, Conjon C: Experimental production of antibodies against stratum corneum keratin polypeptides. Arch Dermatol Res 1980, 267:179–185
17. Dardenne M, Pleau JM, Savino W, Bach JF: Monoclonal antibody against the serum thymic factor. Immunol Lett 1982, 4:79–83
18. Papiernik M, Nabarra B, Bach JF: Monoclonal antibody against the serum thymic factor. Immunol Lett 1982, 4: 79–83
19. Berrih S, Savino W, Azoulay M, Dardenne M, Bach JF: Production of anti-thymulin (FTS) monoclonal antibodies by immunization against human thymic epithelial cells. J Histochem Cytochem 1984, 32:432–438
20. Laemmli VK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 277:680–684
21. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979, 765:4350–4354
22. Henry K: Mucin secretion and striated muscle in the human thymus. Lancet 1966, 1:183–185
23. Rosai J, Levine G: Tumors of the thymus. Armed Forces Institute of Pathology, Washington D.C., 1976

Acknowledgments

The authors thank Drs. Sonia Berrih and Jean-Yves Neveux for providing the thymic fragments, Marie-Claude Gagnerault, Véronique Alves, and Josiane Coulaud for their technical assistance, and Janet Jacobson for reviewing the manuscript.