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Monoclonal Antibodies to *Staphylococcus aureus* Laminin-Binding Proteins Cross-React with Mammalian Cells

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We and others have previously shown that some microorganisms, including bacteria, express on their surfaces receptors that specifically recognize extracellular matrix proteins, such as laminin, fibronectin, or both. The ability of microorganisms to adhere and to invade might depend on the existence of receptors which could, thus, be correlated with pathogenicity. In the present paper, we report the isolation of five stable cell lines that were producers of monoclonal antibodies to *Staphylococcus aureus* laminin receptors. One of these antibodies, which was of the immunoglobulin M isotype, blocked the binding of laminin to bacteria before and after fixation and recognized the putative 52-kilodalton laminin-binding protein in whole bacterial extracts. Also, purified receptor was isolated by immunoaffinity chromatography and shown to bind laminin. Furthermore, the same antibodies bound the 67-kilodalton putative receptor from mouse melanoma cells and gave positive immunofluorescence reactions against mammalian tumor cells. These data strongly suggest either the evolutionary conservation of at least some sequences in both procaryotic and eucaryotic laminin-binding proteins or convergent evolution and positive selection of epitopes cross-reacting with laminin. Some of these antibodies to the procaryotic protein could therefore become useful markers for the expression of laminin receptors by cancer cells.

The ability to bind to basement membranes (BM) has been postulated as an important mechanism through which bacteria and other microorganisms adhere to and invade host tissues (19, 21). Therefore, the recognition of specific ligands on BM could represent a central step in the pathogenicity of organisms (20). BM are thin stromal structures underlying epithelia and are predominantly composed of type IV collagen, proteoglycans, and glycoproteins. Laminin is an 800-kilodalton (kDa) glycoprotein present in BM, which seems to play a fundamental role in cell adhesion and extravasation (24). Specific receptors have been described for metastatic cancer cells (23), granulocytes (3), and lymphocytes (3), as well as macrophages (11), suggesting that the ability to cross the BM is somehow correlated with the expression of surface receptors for laminin. The same rationale led to the demonstration that microorganisms also recognize laminin through specific receptors located on their surfaces. Among those recognizing laminin through surface receptors are some bacteria, namely *Escherichia coli* (19), *Streptococcus pyogenes* (21), and *Treponema pallidum* (7), organisms whose attachment to BM might be important for the colonization of host tissues.

We have recently described and characterized laminin receptors on the surface of a highly invasive bacterium, *Staphylococcus aureus*, but not of the noninvasive *Staphylococcus epidermidis* (15). These receptors can be removed by trypsin and have molecular sizes (approximately 52 kDa) smaller than that of the previously described mammalian 67-kDa receptor (17). Whether the bacterial receptor binds to the same peptide sequence on the laminin molecule, putatively the YIGSR sequence (10) present also on pepsin fragments (P_1) of the molecule (18), remains unknown. One approach to address this question is the production of monoclonal antibodies (MAbs) to either of those binding molecules. MAbs recognizing the eucaryotic receptor have already been produced (13), but their ability to react with bacterial receptors has not yet been demonstrated.

In the present report, we describe the production of five MAbs that specifically recognized *S. aureus* laminin receptors (SAR), one of which blocked the binding of laminin to bacteria and recognized the receptor on whole bacterial extracts. Furthermore, the same MAb also recognized whole mammalian cell extracts and allowed the purification from these cell extracts of a protein with the expected molecular size of 67 kDa.

MATERIALS AND METHODS

Chemicals. Laminin was purified from the mouse Engelbreth-Holm-Swarm tumor as described previously (24). The P_1 fragment was obtained through pepsin digestion of laminin by the method of Rohde et al. (18). Rabbit anti-laminin serum was obtained as previously described (6). CNBr-activated Sepharose and protein A-Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Octyl-β-D-glucopyranoside was from Calbiochem-Behring, La Jolla, Calif. All other chemicals were from Sigma Chemical Co., St. Louis, Mo. Antisera were from Miles Laboratories, Inc., Elkhart, Ind., and conjugates and ^{125}I were from Amersham, England. Bio-Gel P-200 was from Bio-Rad Laboratories, Richmond, Calif.

Bacteria. *S. aureus* Cowan I was a gift from E. P. Camargo, Escola Paulista de Medicina, São Paulo, Brazil. *S. aureus* Wood 46 was a gift from Chun-Yen Lai, Hoffman-La Roche Inc., Nutley, N.J. Both strains were cultivated in Arvidson liquid medium as previously described (16).

Cell culture and production of cell extracts. B16F10 cells were a kind gift from I. Fidler, Houston, Tex.; MG63 and HT1080 cells were from E. Ruoslahti, La Jolla, Calif.; NIH 3T3 and NIH 3T3-ras cells were from B. Groner, LICR, Bern, Switzerland. Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum in a humid atmosphere of 5% CO_2. For the production of extracts, cells were harvested from culture with 0.2% EDTA in phosphate-

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buffered saline (PBS) and washed by centrifugation, and the pellets were treated with equal volumes of 100 mM octyl-β-D-glucopyranoside in PBS (pH 7.4) containing 5 mM calcium and magnesium chloride. After 30 min of incubation, the suspension was again centrifuged and the supernatants were collected. Protein content was measured by the Lowry method.

Purification of SAR. Normal rabbit immunoglobulin G (IgG) was obtained by affinity chromatography on protein A-Sepharose 4B (6). Both rabbit IgG and purified laminin were coupled to CNBr-activated Sepharose according to manufacturer instructions. Whole *S. aureus* extracts were produced from bulk cultures with lysostaphin, as previously described (15). Extracts were repeatedly passed through an IgG-Sepharose column to remove protein A, analyzed before and after by gel electrophoresis and immunoblotting, and then passed through a laminin-Sepharose column. After being washed extensively, bound material was eluted with either low-pH buffer (0.1 M glycine-hydrochloride [pH 3.0]) or with high-salt-plus-detergent solution (1.0 M NaCl, 0.1% octyl-β-D-glucopyranoside). Protein content was measured by the Lowry method, and the material was again analyzed by immunoblotting. SAR thus purified were used both for immunization of mice and as antigens in the screening assays of hybridoma supernatants.

Production and characterization of MAbs. MAbs against purified SAR were made according to a previously described protocol (14). Briefly, BALB/c mice were subcutaneously immunized with 10 μg of SAR in complete Freund adjuvant, followed by three injections in incomplete adjuvant at 3-week intervals. An intravenous booster was made 3 days before fusion. Hybridoma supernatants were screened by an enzyme immunoassay against purified SAR, as described previously (5). After MAbs were cloned by limiting dilution and clones were expanded, MAbs were characterized by immunodiffusion (Ouchterlony) against specific antisera. Ascitic fluid was produced by intraperitoneal injection of 1×10^6 to 2×10^6 cells in pristane-primed mice. MAbs were purified from both ascitic fluid and culture supernatants by ammonium sulfate precipitation and by affinity chromatography on either concanavalin A-Sepharose for IgM isotypes (12) or protein A-Sepharose for IgG isotypes. MAbs of the IgM isotype were also submitted to gel filtration on a column (2.5 by 100 cm) packed with Bio-Gel P-200 equilibrated with PBS (pH 7.4).

Gel electrophoresis, immunoprecipitation, and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described elsewhere (15). Blots were developed with biotinylated second antibodies followed by preformed streptavidin-biotin-peroxidase complexes (Amersham) according to manufacturer instructions. The chromogen used was either diaminobenzidine or 4-chloro-1-naphthol.

Laminin binding. Labeling of bacteria and laminin with ^{125}I, binding, and blocking assays were performed as described earlier (1a, 8, 16).

Indirect immunofluorescence. Cells in culture medium were plated (2×10^4 cells per well) in eight-well tissue culture chamber slides (Lab-Tek; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and incubated overnight. Slides were then washed and dismounted, and the cells were fixed with 0.25% glutaraldehyde. Indirect immunofluorescence reactions were then performed as previously described (1a, 14).

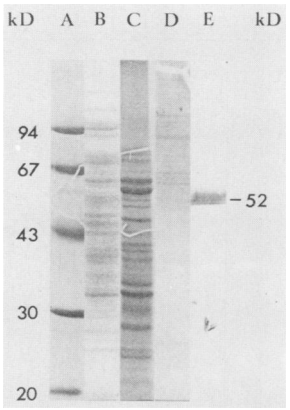

FIG. 1. Affinity purification of *S. aureus* laminin-binding protein. Whole bacterial extract (lane C; SDS-PAGE and Coomassie blue staining) was extensively passed through an IgG-Sepharose column, and the flowthrough material (lane B; SDS-PAGE and Coomassie blue staining) was applied to a laminin-Sepharose column. After being washed extensively, the column was eluted with 1 M NaCl containing 0.1% octyl-β-D-glucopyranoside. Eluted material was transferred to nitrocellulose and sequentially treated with laminin, rabbit anti-laminin, biotin-conjugated anti-rabbit IgG, and streptavidin-biotin-peroxidase complex. The reaction was developed with diaminobenzidine and a single 52-kDa band was revealed (lane E). Lane A, Molecular weight markers; lane D, flow-through material from the laminin-Sepharose column after Coomassie blue staining. Numbers indicate molecular sizes in kilodaltons (kD).

RESULTS

Production and characterization of MAbs. The expression of protein A by *S. aureus*, which binds IgG with high affinity (9), precluded the use of whole bacteria, fixed or unfixed, as antigens for the screening of hybridoma supernatants. This drawback led to the purification of SAR by affinity chromatography after removal of protein A from the whole bacterial extracts. SAR were eluted from the laminin-Sepharose column and, after gel slab electrophoresis in 10% acrylamide, were electrophoretically transferred to a nitrocellulose membrane. Blots were sequentially treated with mouse Engelbreth-Holm-Swarm laminin, rabbit anti-laminin serum, biotinylated sheep $F(ab')_2$ fragment of anti-rabbit immunoglobulin, streptavidin-biotin-peroxidase complex, and diaminobenzidine. A single band of approximately 52 kDa was seen (Fig. 1). This material was considered as purified SAR and was used as antigen for all purposes. Five stable cell lines producing antibodies reactive against SAR were established, four of which were of the IgM isotype (MAbs 1.F4, 1.H12, 7.E7, and 8.H1) and one of which was of the IgG_{2A} isotype (MAb 3.F3). All antibodies were κ light chains. All MAbs were tested for their ability to recognize by immunoblot the same 52-kDa protein which itself bound laminin from whole bacterial protein A-free extracts. With various intensities, MAbs recognized the 52-kDa band (data not shown) after MAb 1.H12 elution by affinity chromatography. All MAbs were then purified, analyzed by SDS-PAGE, characterized by immunodiffusion, and used for all experiments.

Purification of SAR by affinity chromatography with MAb. Whole *S. aureus* extract (20 μg) obtained by treatment with lysostaphin plus detergent was labeled with ^{125}I, mixed with 20 mg (protein content) of total extract, and passed through a MAb 1.H12-Sepharose column. Labeled material was used to monitor column elution. After the column was extensively

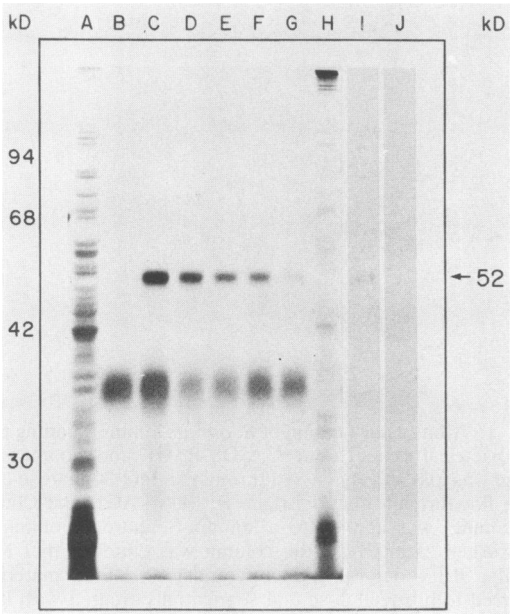

FIG. 2. SDS-PAGE and immunoblot analysis of the affinity purification process of the 52-kDa band with MAb 1.H12. Whole *S. aureus* Wood 46 extract (lane A) was passed through a MAb 1.H12-Sepharose column, and the column was extensively washed (lane H, flowthrough material) and eluted with glycine hydrochloride buffer (pH 3.0). Lanes B through G, Elution tubes 2 to 7. Gels were silver stained after SDS-PAGE analysis. Eluted material was then transferred to nitrocellulose and sequentially treated with laminin and anti-laminin serum as described in the legend to Fig. 1. The 52-kDa protein band is clearly stained (lane I), and no protein is seen when laminin was omitted from the reaction (lane J). The blots in lanes I and J were revealed with 4-chloro-1-naphthol. Numbers indicate molecular sizes in kilodaltons (kD).

washed with PBS (pH 7.4) to remove unbound material, the column was eluted with glycine hydrochloride buffer (pH 3.0). A portion of each fraction was then analyzed by SDS-PAGE with 10% acrylamide, followed by silver staining. A protein band of approximately 52 kDa was seen in the eluted material (Fig. 2). When the band was transferred to nitrocellulose membranes and treated sequentially with laminin and anti-laminin serum, a positive reaction was obtained, showing that the eluted protein binds laminin.

Inhibition of binding of *S. aureus* to laminin. Since *S. aureus* Cowan I expresses large amounts of protein A which could somehow interfere with MAb reactions against whole bacteria, the Wood 46 strain (Sansorbin; Calbiochem-Behring), which does not display protein A on its surface, was used for binding and blocking studies. The amount of laminin receptors expressed by this strain was sufficient to mediate the binding of whole ^{125}I-labeled bacteria to laminin-coated plastic wells (Fig. 3). When MAb 1.H12 was used to coat plastic wells, the binding efficiency of bacteria was similar to that observed with laminin (Fig. 3). With previous incubation of bacteria with MAb 1.H12, binding to laminin was blocked in a dose-dependent fashion. The same binding was not observed when the previous incubation was made with an unrelated MAb (Fig. 4). It is interesting to note that blocking with unlabeled laminin in the same concentrations was always less efficient than blocking with MAbs or with the pepsin fragment P_1 which contains a putative binding sequence recognized by mammalian cell receptors.

Isolation of 67-kDa protein from mouse melanoma cell extracts with MAb. The same procedure used to purify SAR from bacterial extracts was used with B16F10 mouse melanoma cell extracts obtained through detergent treatment.

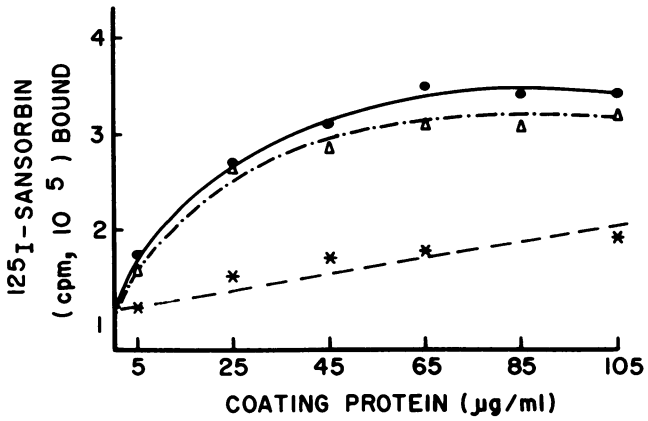

FIG. 3. Binding of ^{125}I-labeled Sansorbin to laminin and MAb 1.H12. A polyvinyl 96-well plate was coated with increasing concentrations of laminin (●), MAb 1.H12 (△), and an unrelated MAb, 4.C11 (*). After the remaining binding sites on plastic were blocked with PBS containing 1% bovine serum albumin, 500,000 cpm of labeled bacteria were added to all wells and incubated overnight. The plate was carefully and extensively washed, and wells were cut and individually counted in a gamma counter. Each point represents the average of triplicate reactions. Binding was observed for both laminin and MAb 1.H12.

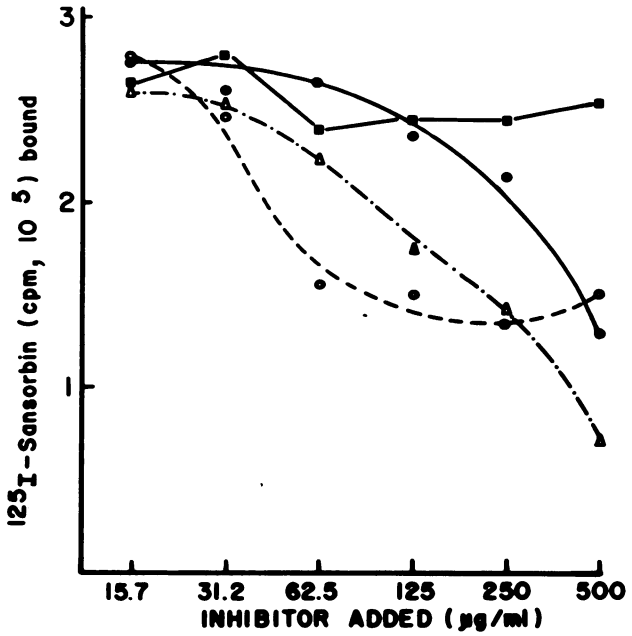

FIG. 4. Inhibition of binding of ^{125}I-labeled Sansorbin to laminin-coated plates. A polyvinyl 96-well plate was coated with 20 µg of laminin per ml in PBS, and the remaining binding sites were blocked with PBS containing 1% bovine serum albumin. Labeled Sansorbin was previously incubated with increasing concentrations of laminin (●), P_1 fragment (△), MAb 1.H12 (○), or the unrelated MAb 4.C11 (■). Bacteria were then added (500,000 cpm per well) to the plate and incubated overnight. The rest of the procedure was as described in the legend to Fig. 3. Each point represents the average of triplicate reactions. Inhibition of binding was dose dependent and more effective with both P_1 fragment and MAb 1.H12.

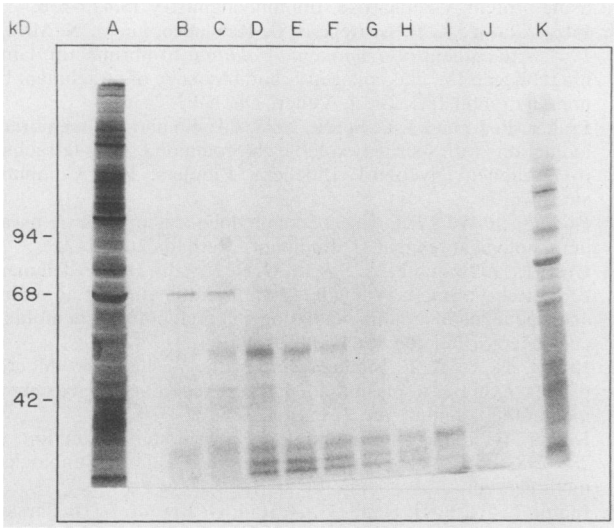

FIG. 5. Affinity purification of the 67-kDa band from mammalian cells (B16 mouse melanoma) with MAb 1.H12. The procedure was as described in the legend to Fig. 2. Elution was made with 4 M magnesium chloride. Lane A, Whole extract; lane K, flow-through material; lanes B to J, elution tubes 2 to 9. Gels were silver stained after SDS-PAGE analysis in 7.5% acrylamide.

After being washed, the column was eluted with 4.0 M magnesium chloride. A portion of each fraction was precipitated with 10% trichloroacetic acid (final concentration), suspended in sample buffer, and analyzed by SDS-PAGE. A sharp 67-kDa protein band was observed (Fig. 5).

Indirect immunofluorescence reactions. Strong immunofluorescence (Fig. 6) was obtained when six different cell lines (Table 1) were treated with MAbs to SAR. No reaction was seen with MAb 5.B3, an unrelated IgM MAb raised against *Schistosoma mansoni* (14).

DISCUSSION

The expression of surface receptors that specifically recognize the protein laminin of BM (22) has been correlated with the capacity of different cells to bind and cross the barrier of BM. Surface receptor expression is thus implicated in both the ability of cancer cells to metastasize (22) and the pathogenicity of microorganisms such as bacteria and protozoa (2, 15). Two different binding sites in the laminin molecule which probably recognize two different proteins on the surface of mammalian cells have already been described (1). Whether the same receptors are responsible for the binding of laminin to phylogenetically different cells remains an unresolved question. One approach to address this issue, as well as to better characterize the binding molecules, is the production of MAbs specific for the receptors. This approach has already been applied in studies with the cancer cell 67-kDa binding protein (13).

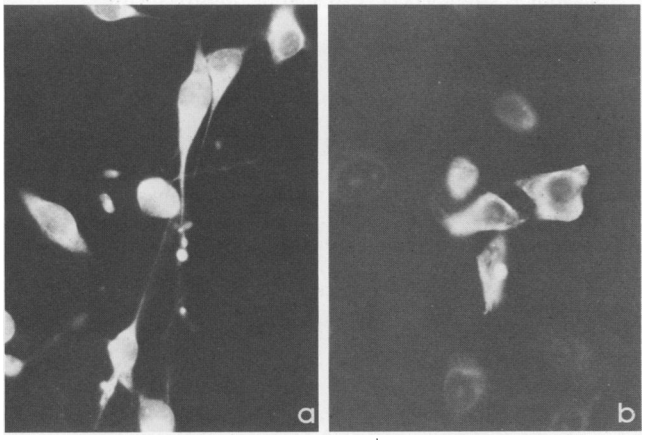

FIG. 6. Typical indirect immunofluorescence reactions of MAb 1.H12 against the cell lines B16 mouse melanoma (a) and MG63 human osteosarcoma (b). No reaction was seen with an unrelated MAb (data not shown). An irregular pattern is seen in panel b, for which only some of the cells were stained.

TABLE 1. Indirect immunofluorescence reactions of MAbs to *S. aureus* laminin-binding protein with human and mouse cell lines

Cell line	Reaction of MAb to *S. aureus* laminin-binding protein[a]		
	1.H12	1.F5F4	7.E7
B16F1	+++	++++	−
NIH 3T3	+++	++++	++++
NIH 3T3-ras	+++[b]	+++	++/+++
Mel 85	+/++	++++	+++
M2R	++/+++	++++	+++
MG63	+/++[b]	++++	++
HT1080	+	++++	−

[a] No reaction was seen with an unrelated IgM MAb (5.B3) produced against *Schistosoma mansoni*. NIH 3T3-ras refers to NIH 3T3 transfected with c-human-*ras*. All reactions were read by three different observers and graded as strongly positive (++++), positive (+++), moderately positive (++), weakly positive (+), and negative (−).

[b] Irregular. Only some cells were stained (Fig. 6b).

In the present paper, we report the production of five MAbs against SAR, four of the IgM isotype and one of the IgG_{2A} isotype. Two of the IgM antibodies (1.H12 and 1.F5F4) recognized the 52-kDa band on bacterial extracts and could be used for affinity purification of the molecule. Also, the thus purified material was recognized by laminin. Since the Cowan I strain expresses large amounts of protein A which migrates in SDS-PAGE in the same molecular weight range as the receptor does (4), all studies with that strain or with the whole cell extract of that strain were precluded because all assays involving immunological development would show unacceptable background. The Wood 46 strain, which does not express protein A but displays laminin receptors (Fig. 3), was then used for most purposes. The results reported here clearly show that *S. aureus* binds well to laminin and the MAbs obtained. Moreover, the binding to the former could be blocked by the latter in a dose-dependent fashion. Similar findings could not be obtained when an unrelated mouse MAb was used (Fig. 4). These results demonstrate that the described MAbs specifically recognized SAR.

An initial indication that the binding site in the laminin molecule recognized by this bacterial receptor could be the same found for mammalian cells came from results with the laminin pepsin fragment P_1. Our results show that P_1 was very effective in blocking the binding of labeled bacteria to laminin-coated plastic wells, even more effective than the whole laminin molecule. This latter finding could be due to the higher solubility of P_1, which does not have the tendency to cluster or to stack on itself. Since P_1 contains the putative binding sequence YIGSR recognized by mammalian cell receptors (1), we decided to determine whether our MAbs could also react with these proteins. The results obtained

with different cancer cell lines strongly suggest that this assumption is correct. MAb 1.H12, for instance, can be used to purify by affinity purification a protein with the expected molecular size of 67 kDa from whole B16F10 mouse melanoma cell extracts. To determine whether that eluted protein is actually the mammalian laminin receptor demands further studies. Indirect immunofluorescence experiments, however, suggested that that could be the case. Strong positive reactions were seen when the MAbs were incubated with different mouse and human cell lines, thus pointing to some evolutionary conservation, convergent evolution, or positive selection of the target antigen. Also, since the profiles of positive indirect immunofluorescence reactions were different for most MAbs studied with different cell lines, it is possible to assume, on the one hand, that the recognized sequences are not the same for all MAbs and, on the other hand, that each target antigen is not expressed by all cells, despite the fact that they all bind laminin.

The MAbs presented here seem to represent, therefore, important reagents in characterizing laminin receptors in different cells and in recognizing sequences that might have been evolutionarily conserved or that might have resulted from positive selection. Also, the results discussed above are encouraging in the sense that these MAbs could be used in screening the expression of receptors by immunohistochemistry and could be valuable tools as tumor markers.

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