Production of polyclonal monospecific antibodies against extracellular matrix (ECM) molecules as matrix research tools

Produção de anticorpos policlonais mono-específico que reconhecem moléculas da matriz extracelular (MEC) como ferramentas de pesquisa

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The extracellular matrix (ECM) is a network of macromolecules which fills the intracellular space. The ECM are composed by a variety of proteins and polysaccharides that are secreted locally by cells in the matrix and assembled into an organized meshwork in close association with the surface of the cell that produced them. At the interface between epithelium and connective tissue, the extracellular matrix forms the basement membranes which in turn form stable sheets through specific self-assembly mechanisms and several of their constituent ligands interact with cellular receptors. Basement membranes are able to determine cell polarity, influence cell metabolism, organize the proteins into adjacent plasma membranes, induce cell differentiation, and serve as specific highways for cell migration (BRENTANI, 1991).

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The ECM is involved in cell properties and activities such as mechanic resistance, elasticity, tissue conformation, cell growth and differentiation, migration, and homeostasis. The cell-matrix interactions have major effects on phenotypic features such as gene regulation, cytoskeletal structure, differentiation, and aspects of cell growth control. The ECM is related to invasive and degenerative processes (MOSHER *et al.*, 1992).

Integrins are the principal receptors used by animal cells to bind to the extracellular matrix. They are heterodimers that function as transmembrane linkers that mediate bidirectional interactions between the extracellular matrix and the actin cytoskeleton. The integrin molecule consists of glycosylated α and β chains held together by noncovalent bonds. Integrins differ from cell surface receptors for hormones and for other soluble signaling molecules in that they bind their ligand with relatively low affinity, a fact that allows the cell moving through the ECM (HUMPHRIES, 1990; VEIGA *et al.*, 1997). They also function as signal transducers, activating various intracellular signaling pathways when activated by matrix binding. A cell can regulate the adhesive activity of its integrins by altering either their matrix-binding site or their attachment to actin filaments. There is a specific tripeptide sequence named RGD (Arg-Gly-Asp) which is found in a number of ECM proteins and is recognized by several members of the integrin family that bind these ECM proteins. Each receptor, however, specifically recognizes its own small set of matrix molecules, indicating that tight receptor binding does not simply require the RGD sequence (HYNES, 1992).

Laminins (LN) are multidomain glycoproteins composed of three polypeptides (α, β and γ chains) that are disulfide bonded into an asymmetric crosslike structure. There are several isoforms of laminin which have a characteristic tissue distribution. Like many other ECM proteins, they consist of a number of different domains that bind proteins and cells

(TRYGGVASON, 1993). Laminin is one of the fist ECM proteins synthesized during embryo development (MINER *et al.*,1997).

Entactin (EN) is a molecule of 150 kDa that plays an essential role in basement membranes assembly. Entactin (nidogen) is a dumbbell-shaped protein that binds tightly to ECM molecules such as laminin, type IV collagen and heparan sulfate proteoglycan, acting as a bridge in basement membrane organization (YAMADA, 1991).

LN and EN were isolated from Engelbreth-Holm-Swarm (EHS) tumors produced intramuscularly in C57-BL10 mice using buffers containing 10 mM EDTA and successive DEAE-cellulose ion exchange chromatography and gel filtration (TIMPL *et al.*, 1979).

ECM components are usually purified from biological materials very rich in a large number of proteins. Thus, the purified extracts may be contaminated with some other proteins even when efficient purification methods are used. SDS-gel electrophoresis applied after purification works as gel filtration, providing a better and more sensitive purification, and the solid polyacrylamide matrix can be used as an adjuvant for the immunological response of the animal.

EHS-LN purified material was eletrophoresed on 5 % polyacrylamide gel in the presence of SDS under reducing conditions and visualized by Coomassie Blue staining. At 440 kD there is the α chain and at 220 kD the β and γ chains that comigrate. Only the α chain was used for animal immunization. Rabbit immunization protocols consisting of 2 intramuscular and 1 subcutaneous applications of about 50 µg protein each, are used for the technique of multiple sensitization points. This method produces a stronger inflammatory response with greater lymphocyte mobilization, thus increasing the probability of a better immune response. The immunizations are performed at 21-day intervals. Blood is collected on the 15th day after the last immunization.

The hyperimmune anti-LN serum collected was evaluated by western-blotting (Figure 1A) and by dot-blotting (Figure 1 B) and the antibody titer found was 1:2000. The protocol for western-blotting and dot-blotting consisted of 2-hour incubation with hyperimmune rabbit serum and 1-hour incubation with anti-rabbit IgG conjugated with alkaline phosphatase, and the enzyme substrate was BCIP-NBT. This polyclonal monospecific antibody also recognizes the protein (LN) in histochemical techniques using EHS-tissue sections (Figure 1D). Figure 1C represents a morphological profile of EHS cells stained by PAS and hematoxylin. The polyclonal LN antiserum specifically reacts with LN among several other molecules tested (FN, VN, Col I, gelatin, BSA, and casein). The antiserum reacts with LN under denaturated conditions (western-blotting assay) as well as recognizes full-lenght molecule (dot-blotting assay).

Fibronectin (FN) is a adhesive protein of ECM, a large glycoprotein found in all vertebrates. This protein is a dimer composed of two very large subunits, named A and B, joined by a pair of disulfide bonds near their carboxyl termini. Each subunit is folded into a series of functionally distinct rodlike domains separated by regions of flexible polypeptide chain. Individuals domains specifically bind to a particular molecule or to a cell. There are multiple isoforms of FN, including one called plasma fibronectin, which is soluble and circulates in the blood and other body fluids, where it is thought to enhance blood clotting, wound healing, and phagocytosis. All of the other forms assemble on the cell surface and are deposited in the ECM as highly insoluble fibronectin filaments. FN is deeply involved in cell migration and proliferation (RUOSLAHTI, 1988; VEIGA *et al.*,1996). FN was purified from fresh human blood plasma by gelatin-sepharose affinity chromatography at room temperature and eluted with 4 M urea (ENGVALL & RUOSLAHTI, 1977).

The purified material was eletrophoresed on 5 % acrylamide gel in the presence of SDS under reducing conditions and stained with Coomassie Blue. The FN dimer, A and B chains,

comigrates at 220 kD. Animals were immunizated with the dimer and the hyperimmune serum collected was submitted to western-blotting (Figure 2A) and dot-blotting (Figure 2B) to evaluate its reaction and specificity. The result was a hyperimmune serum with a positive titer of 1:32000. The antiserum also produced a positive reaction in the hystochemistry assay, using a Sarcoma-180 tissue section (Figure 2D). Figure 2C depicts sarcoma-180 profile stained by Hematoxylin-eosin (HE). The FN polyclonal antiserum showed monospecificity for FN when reacted with other proteins (LN, VN, Col I, BSA, gelatin and casein), and also recognized non-denatured and reduced fibronectins.

EN was purified (PAULSSON *et al.*, 1987) and then electrophoresed on 5 % polyacrylamide gel in the presence of SDS under reducing conditions and visualized by Coomassie Blue staining, with the protein being visualized at 150 kDa. Rabbit immunization protocol was performed at the same experimental conditions as described above.

The hyperimmune serum collected showed specific reaction at a titer of 1:1000 when evaluated by western-blotting (Figure 3A) and dot-blotting (data not shown).

Vitronectin (VN) is a multifunctional serum protein primarily produced in the liver and originally defined as S-protein. VN affects the humoral immune system by binding to and inhibiting the complement C5-9 membrane attack complex. It also serves as a major matrix-associated regulator of blood coagulation (FELDING-HABERMANN & CHERESH, 1993). VN was purified from human blood plasma by heparin-sepharose affinity chromatography, at room temperature, in 8 M urea and β-mercaptoethanol and eluted with 0.5 M NaCl (YATOHGO *et al.*, 1988).

The purified material was eletrophoresed on 10 % acrylamide gel in the presence of SDS under reducing conditions and visualized by Coomassie Blue staining. The vitronectin molecule can be observed at 75 kDa, as intact, and at 65 kDa when hydrolyzed by proteases. The VN polyclonal antiserum is monospecific for this protein and does not

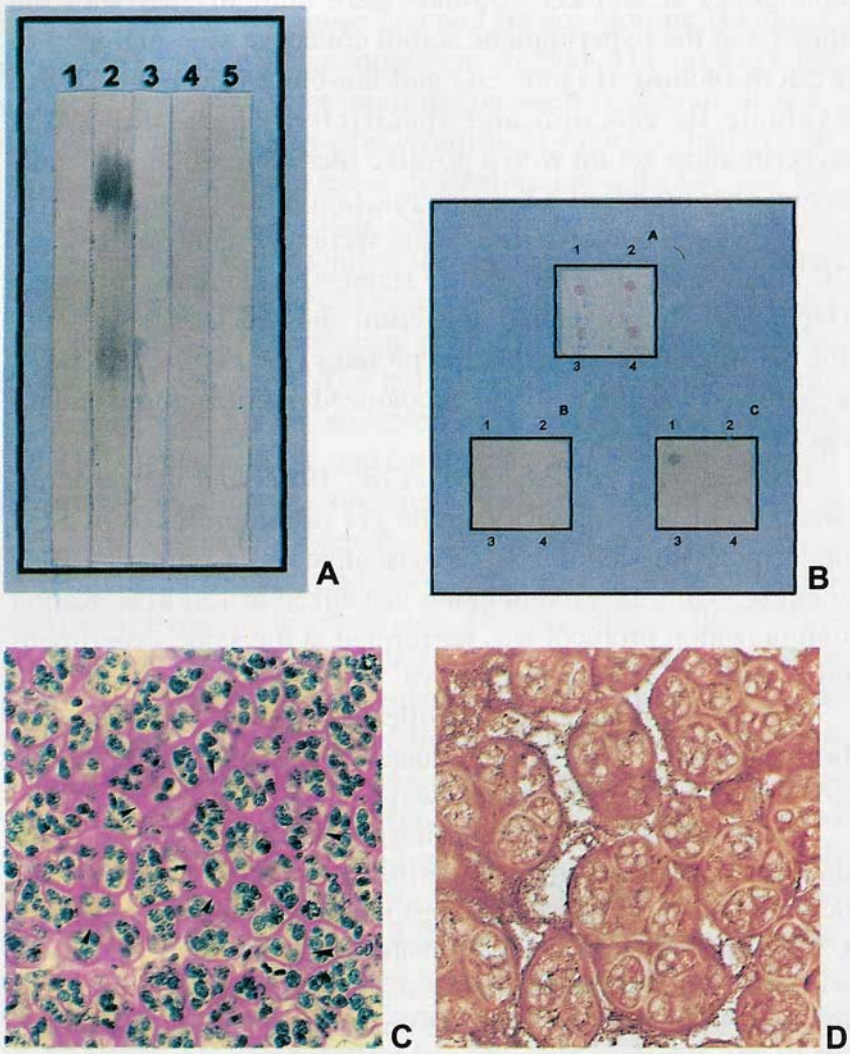

Fig. 1. Reactions of Hyperimmune LN Antiserum. (A) *Western-Blotting Assay*. Line 1- (control) pre-immune serum reacting with laminin (1:2000); line 2- antiserum against LN reacting with purifiед LN (1:2000); line 3- antiserum against LN reacting with FN(1:2000); line 4- antiserum against LN reacting with VN (1:2000); line 5- antiserum against LN reacting with Col I (1:2000). A specific reaction with LN and no cross-reactivity with FN (3), VN (4) Col I (5) were observed. B, *Dot-Blotting Assay*: A, proteins visualized by Ponceau-S staining. Proteins (5 ug): LN (1), bovine serum albumine (BSA) (2), casein (3), gelatin (4); B, (Control) pre-immunc serum 1:2000; C, antiserum against LN 1:2000. Observe the monospecific recognition of laminin by antibodies compared with the other molecules; in this case the molecule is not reduced or denatured. C, *EHS basement membrane pattern*. The figure shows a light micrograph of EHS tumor cells stained with PAS and hematoxylin. Results (PAS positive, arrow heads) point at the tumor capsule which is a thick extracellular matrix of the basement membrane rich in laminin. D, *Immunohystochemistry using hyperimmune LN Antiserum*. EHS tumor tissue fixed with metacan and sectioned for light microscopy was incubated with hyperimmune serum against laminin, diluted 1:500, overnight at 4° C and with secondary goat anti-rabbit IgG peroxidase conjugate (1:50). The reactions were developed with DAB. We can see the laminin stained in the extracellular space, showing a specific recognition of the antiserum tested.

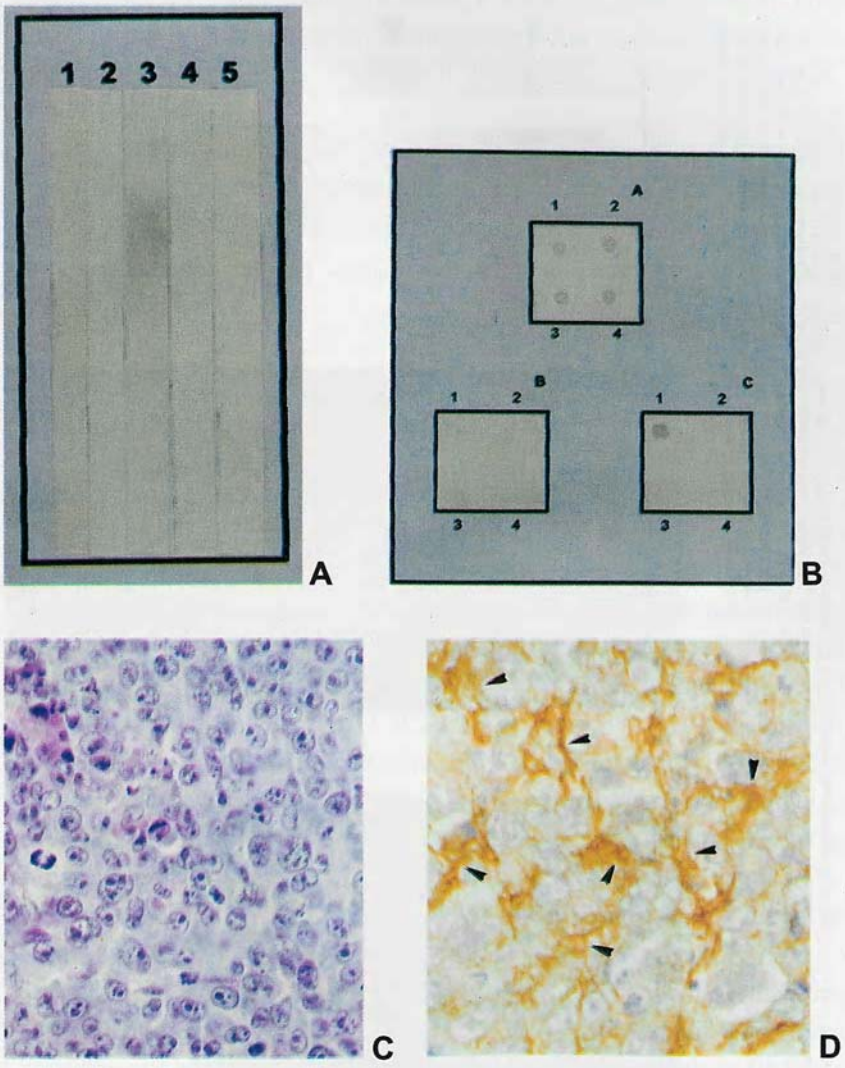

Fig. 2. Reactions of FN Hyperimmune Antiserum. (A) *Western-Blotting Assay*. Line 1, control with pre-immune serum reacting with FN (1:32000); line 2, antiserum against FN reacting with LN (1:32000); line 3, antiserum against FN reacting with FN(1:32000); line 4, antiserum against FN reacting with VN (1:32000); line 5, antiserum against FN reacting with Col I (1:32000). A specific reaction with FN and no cross-reactivity with LN (3), VN (4) or Col I (5) were observed. B, *Dot-Blotting Assay*: A, proteins visualized by Ponceau-S staining. Proteins (5 μg): FN (1), BSA (2), casein (3), and gelatin (4). B, control pre-immune serum, 1:32000; C, antiserum against FN, 1:32000. A monospecific recognition of the FN molecule by antibodies was observed compared with the other molecules; in this case protein is not reduced or denatured. *C, Sarcoma-180 pattern*. Light micrograph of S.180 tissue stained with hematoxylin-eosin. *D, Indirect Immunohystochemistry using Hyperimmune FN Antiserum.* Sarcoma-180 tissue after fixation with metacan and sectioned for light microscopy. The Sarcoma-180 section was incubated sequentially with primary rabbit anti-fibronectin antiserum (diluted 1:500) overnight at 4° C and with secondary goat anti-rabbit IgG peroxidase conjugate (1:50). The reaction was developed with DAB. We can see stained fibronectin in the extracellular space (arrow heads).

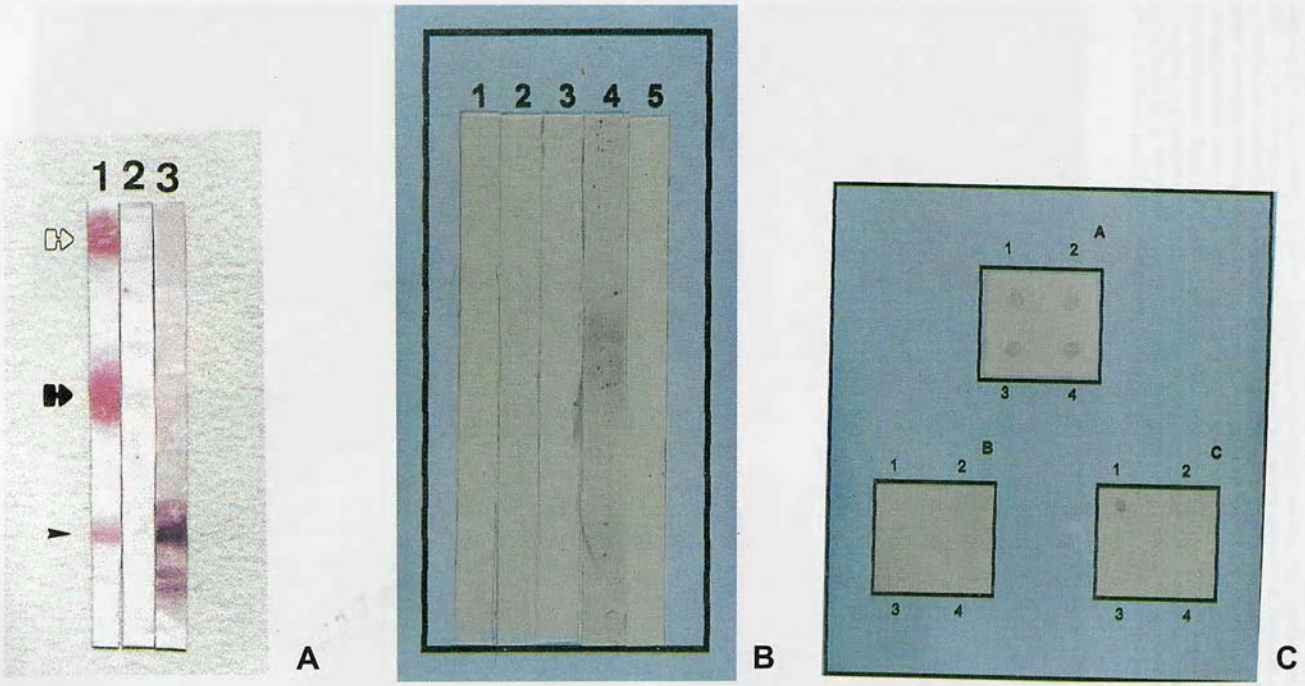

Fig.3. Blotting Assays with Antibodies against Entactin and Vitronectin. A, Western-Blotting with antibodies against EN. Line 1, proteins stained by Ponceau-S (open arrow shows α1 laminin chain, closed arrow β1 and γ1 laminin chains, that comigrate and arrow head entactin; line 2, control with pre-immune serum reacting with LN/EN (1:1000); line 3, antiserum against EN reacting with LN/EN (1:1000). Showing specific reaction with EN and no cross-reaction with LN. B, *Western-Blotting with antibodies against VN.* Line 1, control with pre-immune serum reacting with vitronectin (1:2000); line 2, antiserum against VN reacting with LN (1:2000); line 3, antiserum against VN reacting with FN (1:2000); line 4 , antiserum against VN reacting with VN (1:2000); line 5, antiserum against VN reacting with Col I (1:2000). There is a specific reaction with VN and no cross-reactivity with LN (2), FN (3) or Col I was observed (5). C, *Dot-Blotting with antibodies against VN.* A, proteins visualized by Ponceau-S staining. Proteins (5μg): VN (1), bovine serum albumine (BSA) (2), casein (3), gelatin (4). B, (Control) pre-immune serum 1:2000; C, antiserum against VN 1:2000. Observe the monospecific recognition of VN by antibodies compared with the other molecules; in this case the molecule is not reduced or denaturated.

cross-react with other molecules (LN, FN, Col I, casein, BSA, or gelatin). The polyclonal antiserum against vitronectin reacts with both forms under denatured (Figure 3B) and non-denatured conditions (Figure 3C) at a titer of 1:2000.

These monospecific polyclonal antibodies can be used as biological tools in many scientific procedures and are essential for ECM research protocols.

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RESUMO

A matriz extracelular (MEC) é uma complexa rede de moléculas secretadas que preenchem o espaço extracelular. Biologicamente, a MEC está relacionada com uma grande variedade de funções celulares que refletem a intensa atividade bioquímica desta estrutura. A MEC é composta de moléculas multifuncionais como laminina, entactina, fibronectina, vitronectina, colágenos, proteoglicanos entre outras. O objetivo do presente estudo foi a produção de reagentes associados à matriz extracelular; moléculas purificadas e anticorpos policlonais monoespecíficos. A laminina e a entactina foram isoladas do tumor de Engelbreath-Holm-Swarm (EHS), fibronectina e vitronectina foram purificadas a partir de plasma humano, e o colágeno tipo I foi extraído de tendão de cauda de rato. Após o isolamento, todas estas moléculas da MEC foram submetidas a uma eletroforese SDS-PAGE em gel preparativo e então, injetadas em coelhos usando poliacrilamida ou adjuvante de Freund (completo/incompleto). Após sucessivas imunizações, sangue foi coletado dos animais e a especificidade dos soros hiperimunes avaliadas através de técnicas de western-blotting, dot-blotting e imunohistoquímica. Anticorpos monoespecíficos que reconhecem estas diversas moléculas da matriz extracelular constituem excelentes ferramentas para estudos morfológicos e bioquímicos desta complexa estrutura tecidual.

PALAVRAS CHAVE: ferramentas para pesquisa, matriz extracelular (MEC), anticorpos.

SUMMARY

The extracellular matrix (ECM) is a complex network of secreted macromolecules that fills the extracellular space. Biologically, ECM is related to a large variety of cellular functions which reflect the broad biochemical activity of this structure. ECM is composed of multifunctional molecules such as laminin, entactin, fibronectin, vitronectin, collagens, proteoglycans and others. The objective of the present study was to produce ECM- associated reagents, i.e., purified molecules and monospecific polyclonal antibodies. Laminin and entactin were isolated from Engelbreath-Holm-Swarm (EHS) tumors, fibronectin and vitronectin were purified from human blood plasma, and type I collagen was extracted from rat tail tendon. After isolation, all of these ECM molecules were submitted to preparative SDS-PAGE and then injected into rabbits using polyacrylamide or Freund complete/incomplete adjuvants. After successive immunizations, blood was collected and antibody specifities were checked by western-blotting, dot-blotting and immunohystochemistry techniques. Monospecific antibodies against the major ECM molecules can be excellent tools for morphological and biochemical studies of these complex tissues structures.

KEY WORDS: research tools, extracellular matrix (ECM), antibodies.

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