

Nectadrin, the Heat-stable Antigen, Is a Cell Adhesion Molecule

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Abstract. Nectadrin, the cell surface glycoprotein recognized by the novel mAb 79, was found to be immunologically identical to the heat-stable antigen (HSA). It is a glycoprotein with a polypeptide core of only 30 amino acids and a very high carbohydrate content (Wenger, R. H., M. Ayane, R. Bose, G. Köhler, and P. J. Nielsen. 1991. *Eur. J. Immunol.* 21:1039-1046). Immunocytological studies using cultured splenic B-lymphocytes, neuroblastoma cells, and cerebellar cells indicated that nectadrin is preferentially expressed at sites of cell-cell contact. Purified nectadrin and monoclonal nectadrin antibody 79, but not other monoclonal nectadrin antibodies, inhibited the aggregation of B-lymphocytes by 70%, suggesting that nectadrin may act as a cell adhesion molecule. Nectadrin

was purified from a mouse lymphoma cell line in two forms of 40-60 and 23-30 kD. The lower molecular weight form appears to be generated from the higher molecular weight form by degradative removal of saccharide residues characteristic of complex type oligosaccharide side chains. Latex beads coated with purified nectadrin aggregated and the rate of their aggregation depended on the molecular form of nectadrin, with the larger form being more potent than the smaller one in mediating bead aggregation. Nectadrin thus appears to be a self-binding cell adhesion molecule of a structurally novel type in that its extensive glycan structures may be implicated in mediating cell adhesion.

THE heat-stable antigen (HSA),¹ a cell surface glycoprotein originally defined by mAb M1/69 (Springer et al., 1978) and later also by mAbs J11d (Bruce et al., 1981) and B2A2 (Scollay et al., 1984), has attracted much interest due to its transient, developmentally regulated expression in many cells of haematopoietic lineage. The expression of the heat-stable antigen in B-lymphocytes seems to correlate with their roles in the immune response: its expression is strong in unprimed B-cells and in B-cells that differentiate into antibody-producing cells but is very weak in memory B-cells that have undergone Ig class switching (Takei et al., 1981; Symington and Hakomori, 1984). In T-cell development, those thymocytes that are positive for CD4 and CD8 strongly express the antigen, whereas most CD4⁺CD8⁺ and CD4⁺CD8⁻ thymocytes and peripheral T-lymphocytes show only very weak expression (Takei et al., 1981; Crispe and Bevan, 1987; Husmann et al., 1988). The HSA is also expressed by most myeloid cells and red blood cells throughout their development. However, monocytes cease to express the antigen upon their differentiation into macrophages and maturing erythrocytes acquire a distinct

form of the antigen, lower in its molecular weight than that of lymphocytes and immature monocytes (Miller et al., 1985). The HSA is also expressed in the developing central nervous system and by cultured neurones and neuroblastoma cells (Rougon et al., 1991).

The HSA is a glycoprotein with an extremely small protein core, comprising only 30 amino acids, that in several cell types is anchored to the cell membrane via phosphatidylinositol (Pierres et al., 1987; Alterman et al., 1990; Kay et al., 1990). Additional genes that would account for larger, transmembranal protein cores have also been described, but these forms could not be detected in different tissues and cell lines as gene products (Wenger et al., 1991). The apparent molecular weight of the HSA varies considerably among different cell types but also within each cell type. By Western blot analysis of lymphoid cells the antigen appears as a broad band of 40-68 kD, whereas in mature red blood cells it has an apparent molecular mass of 35-45 kD (Alterman et al., 1990) and in embryonic brain it ranges between 28 and 35 kD (Rougon et al., 1991). After treatment of the cells with tunicamycin as well as after digestion of the antigen with glycopeptidase F to remove N-linked glycans, or with 50 mM NaOH to remove O-linked glycans, the HSA appears by SDS-PAGE as two broad bands of ~19 and 23 kD, suggesting that it is highly glycosylated and heterogeneous with respect to both its N-linked and O-linked carbohydrates (Alterman et al., 1990; Kay et al., 1990).

1. *Abbreviations used in this paper:* DPBS, Dulbecco's modified PBS; h-nectadrin, high molecular weight form of nectadrin; HSA, heat-stable antigen; l-nectadrin, low molecular weight form of nectadrin; LPS, lipopolysaccharide from *Salmonella enteritidis*; MAG, myelin associated glycoprotein; N-CAM, neural cell adhesion molecule.

In view of the developmentally regulated expression of the HSA in haematopoietic and neural tissues, it has been suggested that it may be a differentiation antigen involved in the regulation of lymphoid and neural cell development (Symington and Hakomori, 1985; Alterman et al., 1990; Kay et al., 1990; Rougon et al., 1991). Interestingly, antibody 20C9 to the HSA (but not other mAbs to the HSA) inhibits the CD3 antibody-mediated activation of CD4 T-cells in the presence of lipopolysaccharide (LPS)-activated accessory spleen cells (Liu et al., 1992). CD24 has recently been identified as the human molecule homologous to the murine HSA (Kay et al., 1991). Antibody-mediated cross-linking of CD24 on the cell surface results in increased free intracellular calcium levels in B-lymphocytes and in higher peroxide production in granulocytes (Fischer et al., 1990). CD24 therefore appears to be involved in signal transduction.

In the present study we report that the HSA is a cell adhesion molecule (CAM) in LPS-activated murine B-lymphoblasts. We further show that it has self-binding properties which appear to depend on posttranslational modifications, probably in its glycan structures. In view of its unusual carbohydrate content and ability to mediate cell adhesion we designated it nectadrin.

Materials and Methods

Antibodies

Immunoaffinity-purified rabbit polyclonal antibodies to LI and neural cell adhesion molecule (N-CAM) and preparation of the IgG fraction have been described (Tacke et al., 1987; Kadmon et al., 1990a,b). mAbs M1/69 (Springer et al., 1978; IgG_{2b}), B2A2 (Scollay et al., 1984; IgM), and J11d (Bruce et al., 1981; IgM), recognizing the HSA, were kind gifts from M. Simon (Max Planck Institute for Immunobiology, Freiburg, Germany), H. von Boehmer (Basel Institute for Immunology, Basel, Switzerland), and M. Pierres (Centre of Immunology, INSERM-CNRS de Marseille, Marseille, France), respectively. mAb FD18.5, recognizing the lymphocyte function-associated antigen 1 (LFA-1; Sarmiento et al., 1982; IgG_{2a}) also was a gift from M. Simon. mAbs 30-H12, recognizing Thy-1 (Ledbetter and Herzberg, 1979), and RA3-6C2, recognizing the B220 antigen of B-lymphocytes (Coffman and Weissman, 1981), were kind gifts from B. Kyewski (The German Cancer Research Centre, Heidelberg, Germany). mAb 12.15, recognizing CD2 (Altevogt et al., 1988, 1989a; IgG₁) has been described. Nonspecific rat IgG antibodies were purified on a protein G column (Pharmacia, Uppsala, Sweden) from sera of nonimmunized adult rats. They included IgG₁, IgG_{2a}, and IgG_{2b} isotypes as tested by enzyme-linked immunosorbent assay (ELISA) with subclass-specific antibodies (Dianova, Hamburg, Germany) (results not shown).

Secondary antibodies against rat and rabbit immunoglobulins, conjugated to alkaline phosphatase (Sigma Chemical GmbH, Munich, Germany) or to fluorescein isothiocyanate (FITC; Dianova) as well as streptavidin conjugated to fluorescein isocyanate or phycoerythrin (Boehringer Mannheim GmbH, Mannheim, Germany) were purchased from the commercial sources. The conjugation of primary antibodies to biotin or FITC was done according to Kadmon et al. (1990b).

Production of Antibodies to Nectadrin

mAb 79 to nectadrin (IgG_{2a}; Altevogt et al., 1988, 1989a) was obtained from the fusion of spleen cells from a rat immunized with crude membrane preparations from ESb 289 tumor cells (Schirmacher et al., 1979, 1982; Altevogt et al., 1988, 1989a). It was purified from the hybridoma supernatant on a Protein G column. Polyclonal antibodies to nectadrin were obtained by immunizing a rabbit with biochemically purified nectadrin (see below; Fig. 1) and purification of the IgG fraction of the antiserum over a protein A column (Pharmacia). The immunization procedure followed established protocols (Shiigi and Slomich, 1980), using 100 µg of the purified antigen for the initial immunization in complete Freund's adjuvant and for each of the following three boosts in incomplete Freund's adjuvant. By Western blot analysis, both mAb 79 and the polyclonal antibody recognize

purified nectadrin (for mAb 79, see Fig. 6) and, when using crude membrane preparations from ESb 289 cells, a broad band that comigrates with purified nectadrin (not shown). Both antibodies also recognize the bacterial fusion protein of the HSA (Wenger et al., 1991).

Antigens

The isolation of LI (Rathjen and Schachner, 1984), N-CAM (Thiery et al., 1977), and the myelin associated glycoprotein (MAG; Salzer et al., 1987) as integral membrane proteins (designated detergent-soluble form) has been described (Kadmon et al., 1990a). By Western blot analysis, these preparations did not contain bands that could be recognized by antibodies to nectadrin (not shown). For coating latex beads, the proteolytic fragments containing the extracellular domains of these integral membrane proteins (designated soluble form; Kadmon et al., 1990a) were used. Recombinant CD2 fusion protein containing the two extracellular domains of murine CD2 and the constant part of the murine κ -light chain produced in murine myeloma cells (Rutschmann and Karjalainen, 1991) was a kind gift from R. Rutschmann (Basel Institute for Immunology, Basel, Switzerland).

Purification of Nectadrin

Nectadrin was either purified by ion exchange and gel filtration chromatography or by immunoaffinity chromatography using mAb 79. For the former (Fig. 1), sucrose density gradient-purified crude plasma membrane preparations (Thinnes et al., 1984) from ESb 289 cells (10 g wet weight; a kind gift of N. Hilschmann, Max Planck Institute for Experimental Medicine, Göttingen, Germany) were solubilized in 50 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 2% CHAPS (both from Sigma Chemical GmbH). After centrifugation at 100,000 g for 1 h, the supernatant was collected and dialyzed against the same buffer containing 0.2% CHAPS. The supernatant was then applied to a Mono Q column (Pharmacia) and eluted with a 20–1,000 mM NaCl gradient in this CHAPS-containing buffer. The first protein peak (Fig. 1 A) was recovered and dialyzed against 25 mM diethanolamine-HCl, pH 9.3, 20 mM NaCl, and 0.2% CHAPS. It was then applied to a Mono P column (Pharmacia) and eluted as described for the Mono Q column, but using a 20–500 mM NaCl gradient in the latter buffer. The first protein peak (Fig. 1 B), containing mAb 79-reactive material, was again recovered, dialyzed against 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, and 0.2% CHAPS, and applied to a Superose 6 column (Pharmacia) for size fractionation. The main protein peak eluted (Fig. 1 C) contained purified nectadrin which migrated by SDS-PAGE as a broad band of 40–60 kD (Fig. 1 D).

Immunoaffinity purification of nectadrin from sucrose density gradient-purified crude plasma membrane preparations (Thinnes et al., 1984) from the lymphoma cell line ESb 289 was performed according to Rathjen and Schachner (1984) using mAb 79 to nectadrin coupled to Sepharose 4B. Nectadrin was eluted from the column with a 0.1 N diethylamine buffer, pH 11.5, containing 0.1 N NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mg/ml deoxycholic acid (DOC) and was thoroughly dialyzed against 20 mM Tris-HCl, pH 8.0, containing 20 mM NaCl and 1 mg/ml DOC. This preparation yielded a molecular form of nectadrin with a minor broad band of 40–60 kD and a major broad band of 23–30 kD (see Fig. 6) as seen by SDS-PAGE and Western blot analysis (Towbin et al., 1979). The two forms of nectadrin, the form purified by ion exchange and gel filtration chromatography, with the higher apparent molecular weight, and the form purified by immunoaffinity chromatography, predominantly with the lower apparent molecular weight, will be referred to as h-nectadrin and l-nectadrin, respectively. No impurities were found in the nectadrin preparations by SDS-PAGE after staining by the reducing silver method (for h-nectadrin, see Fig. 1 D) or by Western blot analysis using polyclonal antibodies to LI or N-CAM or mAb 12.15 to CD2 (not shown).

Deglycosylation

To remove N-linked glycans from nectadrin, nectadrin was precipitated with acetone overnight at 20°C and dissolved in 50 µl endoglycosidase buffer (100 mM Tris-HCl, pH 8.0, containing 10 mM EDTA, 5 mg/ml SDS, and 140 mM 2-mercaptoethanol), and heated for 3 min to 95°C. Nectadrin was then incubated overnight at 37°C in the presence of 0.2 U endoglycosidase F/N-glycopeptidase F (Boehringer Mannheim GmbH; Tarentino et al., 1985) and 1% (vol/vol) Triton X-100 (Sigma Chemical GmbH). The digestion was terminated on ice and by the addition of electrophoresis sample buffer (Laemmli, 1970) and boiling for 3 min. For control, the enzyme was omitted.

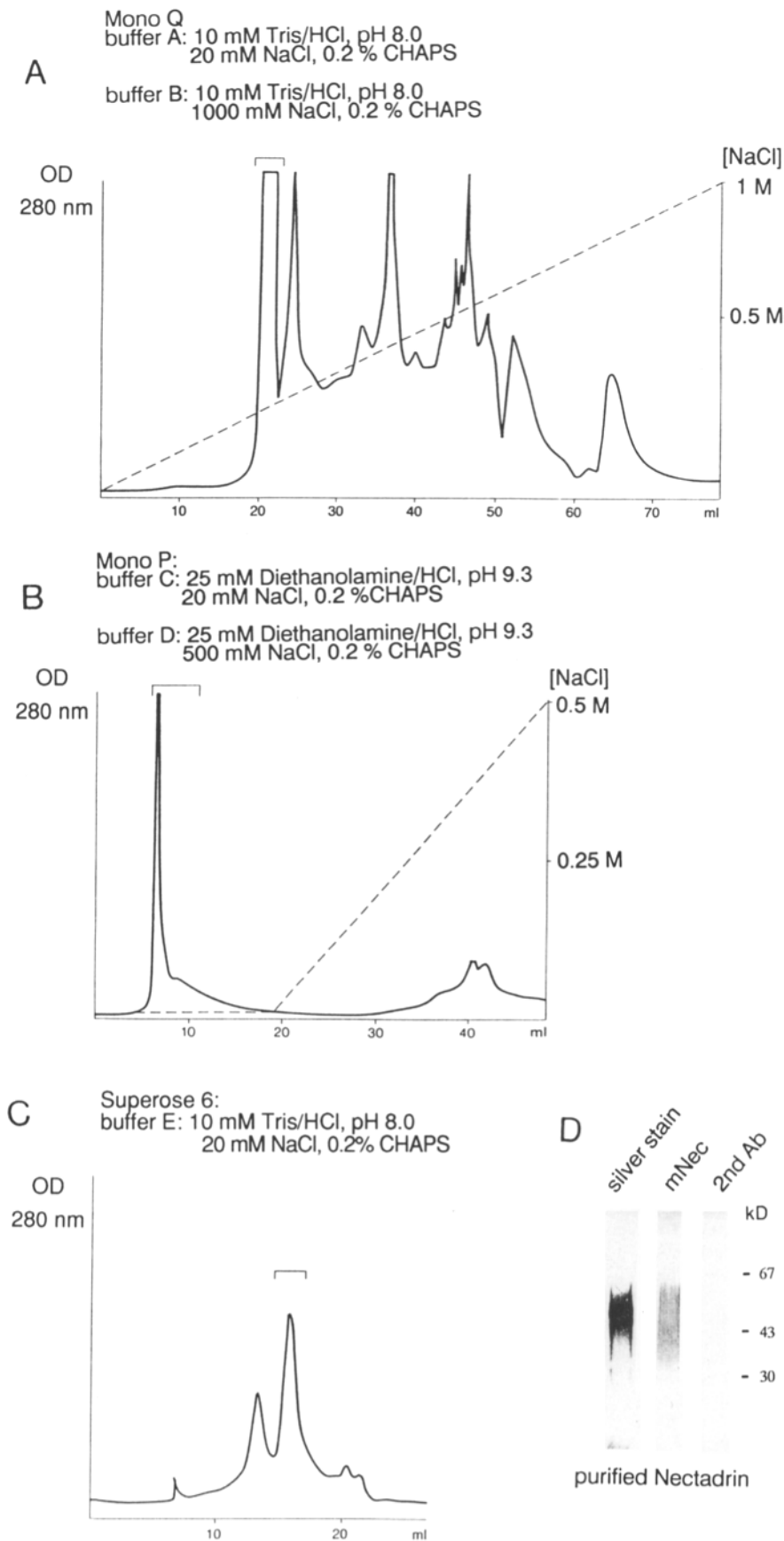


Figure 1. Ion exchange and gel filtration chromatographic purification of nectadrin. (A) ESb 289 cell membrane preparations were solubilized in buffer A, applied to a mono Q anion exchange column and eluted with a continuous gradient of buffer A to buffer B. The first eluted protein peak (marked by a *bracket*) included nectadrin-positive material and was used for further purification. (B) The isolated first protein peak in A was dialyzed against buffer C, applied to a mono P cation exchange column and eluted with a continuous gradient of buffer C to buffer D. The first eluted protein peak (marked by a *bracket*) again contained nectadrin-positive material and was used for further purification. (C) The isolated first protein peak in B was dialyzed against buffer E and separated on a Superose 6 size fractionation column. The major protein peak (marked by a *bracket*) eluted after chasing the loaded sample with buffer E was further analyzed. (D) The isolated protein peak in C (marked by a *bracket*) was tested by SDS-PAGE followed by silver staining (*silver stain*) and Western blot analysis with mAb 79 (*mNec*). For control, Western blot analysis was performed omitting the primary antibody (*2nd Ab*). The positions of molecular mass markers are indicated in kD at the right margin (*kD*).

Lectin Binding Analysis

A mixture of the two forms of nectadrin as well as the control glycoproteins transferrin, fetuin, asialofetuin, and carboxypeptidase Y (all from Boehringer Mannheim GmbH) were electrophoretically separated and transferred to nitrocellulose sheets as for Western blot analysis (see below). The ability of digoxigenin-labeled agglutinins from *Datura stramonium*, *Maackia amurensis*, *Galanthus nivalis*, *Sambucus nigra*, *Aleuria arantia*, and *Ulex europaeus* (Glycan Differentiation Kit; Boehringer Mannheim GmbH) as well as concanavalin A to bind to these proteins was then tested according to the instructions of the manufacturer.

Biosynthetic Carbohydrate Labeling and Immunoprecipitation

For biosynthetic labeling with radioactive sugars, 10^7 ESb 289 cells were incubated for 24 h at 37°C in complete RPMI 1640 medium (Boehringer Mannheim GmbH) supplemented with 50 μ Ci/ml of each 3 H-labeled fucose, glucosamine, and galactose (Amersham Buchler, Braunschweig, Germany) as previously described (Altevogt et al., 1989a). The cells were then washed and lysed in the presence of 1% Triton X-100. Nectadrin was subsequently immunoprecipitated using a mixture of mAbs 79, B2A2, M1/69, and J11d as previously described (Lang et al., 1987). The immunoprecipitate was separated electrophoretically and examined by autoradiography as previously described (Lang et al., 1987).

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8, 10, or 15% slab gels containing an acrylamide/bisacrylamide ratio of 30:0.8 using the Laemmli buffer system (Laemmli, 1970). Western blot analysis was performed according to Towbin et al. (1979). When detergent-solubilized cells were analyzed, the cells were homogenized in hypotonic Tris-HCl buffer (10 mM, pH 7.4) containing 1% Triton X-100, cleared by centrifugation (100,000 g, 30 min), and mixed with an equal volume of 2 \times concentrated electrophoresis sample buffer (Laemmli, 1970) before boiling them for 3 min.

Cells and Cell Culture

Spleen cells were obtained from 6-wk-old DBA/2 mice. They were aseptically removed from their connective tissue capsule with a bent canule, washed in PBS, pH 7.2 (100 g for 10 min at 4°C), treated with 0.15 M NH_4Cl , 0.017 M Tris-HCl, pH 7.2, to lyse erythrocytes (Mishell and Shiigi, 1980), and washed twice again in PBS. They were maintained in tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS. To enrich the primary cultures for B-lymphocytes, B-lymphocytes were selectively induced to proliferate by the addition of LPS from *Salmonella enteritidis* (50–100 ng/ml; Serva, Heidelberg, Germany) to the cultures for 2 d (Morrison and Ryan, 1979). For immunofluorescence microscopy, B-lymphocytes were cultured as monolayers on poly-L-lysine-coated glass coverslips under the same conditions. For cell aggregation assays, LPS-activated B-lymphocytes were further purified by Ficoll-Paque 400 (Pharmacia) gradient centrifugation according to the instructions of the manufacturer. The purity of the cell preparations for B-lymphocytes was examined by cytofluorography using mAbs to the B220 antigen (Coffman and Weissman, 1981) and Thy-1 (Ledbetter and Herzberg, 1979) and generally exceeded 90%. Thymocytes were obtained from thymus of 6-wk-old DBA/2 mice as previously described (Altevogt et al., 1989a). Bone marrow cells and erythrocytes were obtained from the tibia (Whitelock et al., 1984) and blood of DBA/2 mice, respectively.

The cell lines, Eb 288 and ESb 289 (T-lymphoma; Schirrmacher et al., 1979), EL-4 (T-lymphoma; Culvenor et al., 1981), WEHI 267.5 (plasmocytoma; Culvenor et al., 1981), 18-81 (pre-B-lymphoma; Culvenor et al., 1981), P3x63Ag8.653 (plasmocytoma; Kearney et al., 1979), and BALB/3T3 (fibroblast cell line; Aaronson et al., 1968) have been described. They were maintained in RPMI-1640 medium containing 10% FBS.

Cerebellar cells were obtained from 8-d-old NMRI mice by dissociation with Dispase II (Boehringer Mannheim GmbH) as described (Faissner et al., 1984). The cells were maintained on poly-L-lysine-coated coverslips for 16 h or 3 d in basal medium Eagle's supplemented with 10% horse serum and 50 μ g/ml gentamycin (Sigma Chemical GmbH). N2A neuroblastoma cells were maintained in cell culture bottles in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% FBS (Rathjen and

Schachner, 1984), or they were maintained in the same culture medium on poly-L-lysine-coated glass coverslips for 3 d.

Immunofluorescence Cytology

To localize nectadrin on the surface of cultured cells, indirect immunofluorescence cytology was performed as described by Schnitzer and Schachner (1981). Coverslips carrying the cells were incubated for 1 h in 50 μ l mAb 79-conditioned hybridoma culture medium. For control, unconditioned medium and 100 μ g/ml polyclonal rabbit antibodies to liver membranes were also used. The cells were then washed in DPBS, fixed for 15 min in 4% paraformaldehyde in DPBS, washed again in Dulbecco's modified PBS (DPBS) (includes Ca^{2+} and Mg^{2+}), and incubated for 15 min in DPBS containing 1% BSA. They then were incubated for 20 min with FITC-conjugated secondary antibodies to rat immunoglobulins diluted 1:100 in DPBS. All procedures were performed at room temperature.

Cytofluorographic Analysis

The expression of cell surface antigens was quantitated by indirect immunofluorescence using a fluorescence activated cell sorter (FACScan with LYSYS II, Becton Dickinson Immunocytometry Systems, Mountain View, CA) as described (Lang et al., 1987). One million cells were harvested, washed twice in PBS, and incubated for 1 h with biotinylated mAb 79 to nectadrin at a concentration of 25 μ g/ml, washed in PBS, and further incubated for 30 min with phycoerythrin-conjugated streptavidin (Sigma Chemical GmbH; 10 μ g/ml). FITC-conjugated mAbs to the B220 antigen and to Thy-1 (25 μ g/ml) were then added to the cells after the cells had been thoroughly washed. The cells were washed again, resuspended in 500 μ l PBS, and analyzed by cytofluorography. The mean relative fluorescence intensity per cell was evaluated from samples of 10,000 cells. All procedures were performed on ice. The content of nectadrin on antigen-coated latex beads was examined by the same procedure.

Coating of Latex Beads with Glycoproteins

Latex beads were coated with nectadrin or with the soluble form of L1 or MAG (see above) as described (Kadmon et al., 1990a). To this end, DOW 7 latex beads (Serva) with a 7- μ m mean diameter were used. For coating, 150 μ l of a 10% bead suspension were mixed with a glycoprotein solution containing 20 μ g detergent-soluble nectadrin, 30 μ g soluble L1, or 30 μ g soluble MAG (55–100 μ g/ml for each glycoprotein). L1 and MAG were coated by an overnight incubation in an Eppendorf Mixer 5432 (Kurt Migge, Heidelberg, Germany) at 4°C. Detergent-soluble nectadrin kept in Tris-HCl, pH 7.2, containing 0.2% CHAPS (for h-nectadrin) or in Tris-HCl buffer, pH 8.0, containing 0.1% deoxycholic acid (for l-nectadrin) was coated on a reciprocal shaker at 4°C during dialysis against PBS, pH 7.2, for 3 d with nine changes of the dialysis buffer according to Kadmon et al. (1990a). For detergent-soluble antigens this coating procedure was chosen since the coating efficiency was highest when the detergent was removed from the antigen solution in the presence of the beads (not shown).

The coating efficiency was examined by Western blot analysis (not shown). For this purpose, the glycoprotein-coated beads were sedimented in the coating solution by centrifugation (5 min, 8,000 rpm in a Biofuge A centrifuge, Heraeus Sepatech, Osterode am Harz, Germany) and 30 μ l of the supernatant were removed and mixed with 10 μ l of a 4 \times concentrated electrophoresis sample buffer (Laemmli, 1970). The sedimented beads were then resuspended and 30 μ l of the suspension were removed and recentrifuged. The pelleted beads were thoroughly washed in PBS and resuspended in 40 μ l electrophoresis sample buffer. The presence of the glycoproteins in the supernatant and bead fractions was examined by Western blot analysis using polyclonal antibodies and applying the corresponding bead and supernatant fractions to adjacent lanes. After adding 7 μ g nectadrin, 10 μ g L1, or 10 μ g MAG to the beads (approximately 8.5×10^5 , 5.0×10^5 , and 7.5×10^5 molecules per bead for nectadrin, L1, and MAG, respectively), all of the glycoproteins were associated with the beads in that no remaining glycoproteins were detectable in the supernatant after coating. When 20 μ g nectadrin or 30 μ g L1 or 30 μ g MAG were used ($\sim 2.5 \times 10^6$, 1.5×10^6 , and 2.2×10^6 molecules per bead for nectadrin, L1, and MAG, respectively), the beads were saturated with respect to the coating protein and had no additional free protein binding sites in the sense that beads coated with nectadrin (nectadrin beads) or L1 (L1 beads) could not further bind MAG and MAG beads had no remaining free binding sites for nectadrin or L1. By cytofluorographic analysis the beads showed strong and uniform expression of the coating antigens that was not reduced by preincubat-

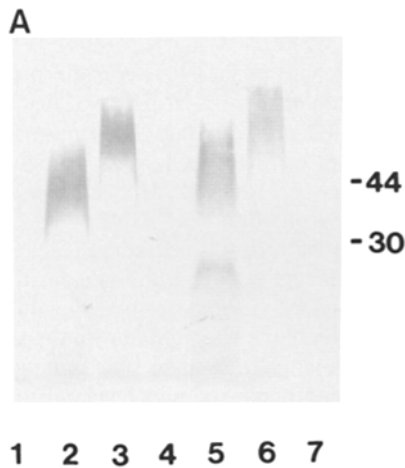
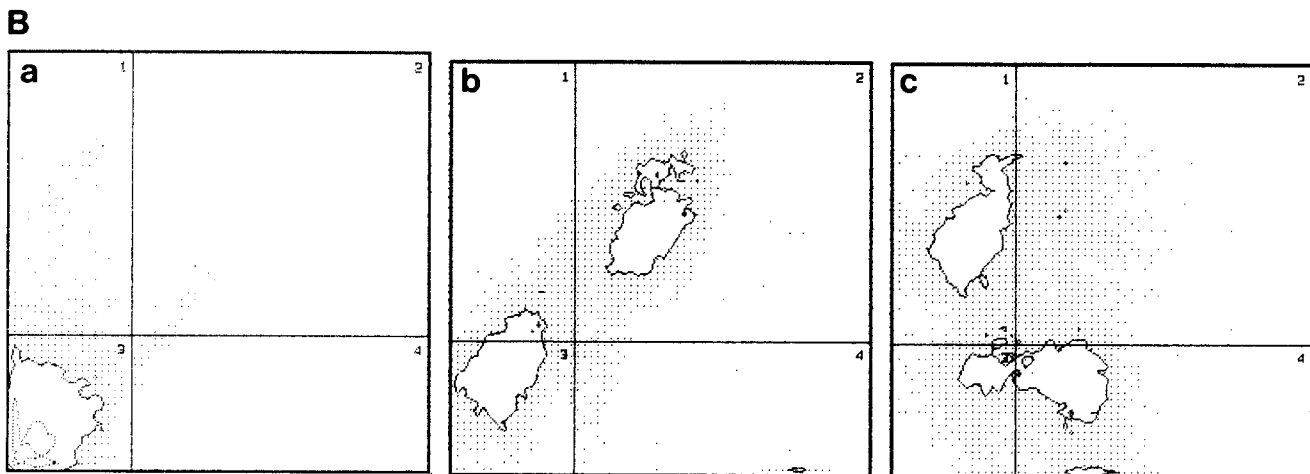


Figure 2. Determination of the expression of nectadrin by different cell lines and splenic B-lymphocytes. (A) Western blot analysis of the presence of nectadrin in different cell lines. Detergent-solubilized lysates of the cell lines BALB/3T3 (lane 1, fibroblast cell line); P3x63Ag8.653 (lane 2, plasmocytoma); 18.81 (lane 3, pre-B-cell line); EL-4 (lane 4, T-lymphoma); ESb 289 (lane 5, T-lymphoma); WEHI 267.5 (lane 6, plasmocytoma); and Eb 288 (lane 7, T-lymphoma) were separated by SDS-PAGE, transferred to nitrocellulose sheets, and allowed to react with mAb 79 to nectadrin by Western blot analysis. The positions of molecular weight markers are indicated in kD at the right margin. (B) Two-color cytofluorographic examination of the expression of nectadrin by B-lymphocytes in the spleen. Dissociated mouse spleen cells that include B- and T-lymphocytes were incubated with phycoerythrin-conjugated streptavidin only (a) or with biotinylated rat mAb to nectadrin, visualized by phycoerythrin-conjugated streptavidin (b and c). The cells were then incubated with no further antibodies (a) or with FITC-conjugated rat mAb to the B-lymphocyte marker antigen B220 (b) or FITC-conjugated rat mAb to the T-lymphocyte marker antigen Thy-1 (c). Ordinate and abscissa denote the relative fluorescence intensity per cell measured for phycoerythrin and FITC, respectively. Results are from one representative experiment using 10,000 cells per sample.



ing the beads with 1 mg/ml BSA. The mean fluorescence intensity per bead was compared with the mean fluorescence intensity per activated B-lymphoblast tested in parallel using the same antibodies and measured under the same adjustment of parameters in the fluoroscanner.

Homotypic Aggregation of Cells and Glycoprotein-coated Latex Beads

The aggregation of cells and of glycoprotein-coated latex beads was tested as described (Kadmon et al., 1990a). LPS-activated B-lymphocytes were suspended in PBS, pH 7.2, containing 2 mg/ml EDTA and beads were resuspended mechanically by using a Vortex mixer. Both cells and beads were further washed in PBS, pH 7.2, before the aggregation test. For the analysis of the aggregation, 25 μ l of a cell suspension containing \sim 100,000 cells or 10 μ l of a bead suspension containing \sim 300,000 beads were added to Ca^{2+} and Mg^{2+} -free HBSS or DPBS in 24-well tissue culture plates (Costar Corp., Cambridge, MA) to a final volume of 300 μ l per well. For cells, the wells had been precoated with PBS containing 100 mg/ml heat-treated (75°C, 20 min) BSA to prevent the adhesion of the cells to the plastic surface. When antibodies or antigens were added to the cells or beads, they first were incubated with the cells or beads for 30 min on ice to allow for good binding. For cells, antibodies and antigens were added to a final concentration of 100 and 20 μ g/ml, respectively. For beads, the concentrations of antibodies and antigens were 35–70 μ g/ml and 10 μ g/ml, respectively. For all experiments, it was ascertained by titration that at these concentrations the antibodies and antigens produced their maximal inhibitory effect (not shown). mAb M1/69 and the polyclonal antibody to nectadrin were additionally tested in the cell aggregation assay at concentrations of up to 10 mg/ml.

For cell and bead aggregation, the culture plates were incubated at 37°C and at room temperature, respectively, at a rotation speed of 85 rpm for the durations specified in the figure legends. Aggregation was stopped by placing the culture plates on ice and by dilution of the cells and beads in the

Coulter Counter electrolyte solution. The degree of aggregation was evaluated microscopically and by Coulter Counter analysis (see below). All aggregation experiments were performed in triplicates or in hexaplicates.

Heterotypic Aggregation of Glycoprotein-coated Latex Beads

In addition to the homotypic aggregation of latex beads coated with one glycoprotein or with a mixture of two glycoproteins, the heterotypic aggregation of beads coated with different glycoproteins was also examined. For the analysis of heterotypic aggregation, the differently coated beads were added either to the same well, where they could coaggregate, or to two separate wells, in which case they were mixed together for measurement only after their aggregation had been terminated. The difference in particle aggregation seen with the two procedures as evaluated by Coulter Counter analysis was taken to represent the heterotypic aggregation (coaggregation) of the differently coated beads (see Fig. 8; see also Kadmon et al., 1990a). All aggregation experiments were performed in triplicates or in hexaplicates.

Coulter Counter Analysis of Aggregation

The aggregation of cells and beads was quantitated in a Coulter Counter model T_{ALL} fitted with a Population Accessory using a nozzle 200 μ m in diameter (Coulter Corp., Hialeah, FL). For the analysis of aggregation, the distribution of partial volume (vol %) over the different channel numbers was used. The channel numbers correspond to particle size, that is, to the size of single cells or beads and to the sizes of their aggregates. The vol % value read for each channel number corresponds to the percent of all cells or beads that are present in particles of the size measured in the respective channel number. The inhibition of aggregation was evaluated from the reduction in the cumulative vol % value corresponding to large aggregates

when the aggregation had been performed in the presence of additives as compared to the aggregation in the absence of additives.

For the evaluation of the heterotypic aggregation of differently coated beads, the vol% distribution curves obtained when the differently coated beads had been mixed together prior to and after aggregation were compared (see Fig. 8 B). Additionally, the total number of particles per well (N) before and after aggregation was also evaluated (Fig. 8 C). Before aggregation, N is equal to the number of single beads added to each well and is equal for all wells. The number of particles, N, then decreases in proportion to the degree of aggregation (Fischer and Schachner, 1982).

Statistical Analysis

For statistical evaluation of the results, one way nested analysis of variance (nested anova) and multiple comparisons among means using the GT2 and Student-Neuman-Keuls methods with $\alpha \leq 0.01$ were performed. All the statements in this work concerning differences between the quantitative effects of different treatments are based on one of these multiple comparison tests but reference to the actual test has been omitted.

Results

Characterization of a Novel mAb to the HSA Designated Nectadrin

mAb 79 resulted from the immunization of a rat with plasma membranes from the lymphoma cell line ESb 289 (Altevogt et al., 1989b; see also Fig. 2 A). The antibody reacted by indirect immunofluorescence and Western blot analysis also with other established mouse tumor lines of haematopoietic (P3x63Ag8.653, 18-81, and WEHI 267.5; Fig. 2 A) origins as well as with normal lymphoid and neural cells (see Fig. 4). The apparent molecular weights of the antigen recognized by mAb 79 varied between 30 and 68 kD, depending on the cell type (Fig. 2 A). The broad size distribution was also noted when lysates from normal thymocytes, B-lymphocytes, and erythrocytes were examined (not shown). In ESb 289 cells, two immunoreactive bands were seen of 23 and 40–60 kD (Fig. 2 A, lane 5). The binding of mAb 79 to thymocytes could partially be blocked by mAbs M1/69, B2A2, and J11d to the HSA but not by antibodies to CD2 (not shown). It has recently been shown that mAb 79 as well as the polyclonal nectadrin antibody recognize the bacterial fusion protein of the heat-stable antigen (Wenger et al., 1991).

Characterization of High and Low Molecular Weight Forms of Nectadrin

To study the functional properties of the isolated molecule, nectadrin was purified from plasma membranes of ESb 289 lymphoma cells. Two forms of nectadrin were isolated from these cells, one of 40–60 kD, designated h-nectadrin (Fig. 3 A, lanes 1–5), and one of 23–30 kD, designated l-nectadrin (Fig. 3 A, lanes 6–10). h-nectadrin was isolated biochemically as outlined in Fig. 1. Immunoaffinity purification using monoclonal antibody 79 predominantly yielded l-nectadrin and, to a minor extent that varied from batch to batch, also h-nectadrin. L-nectadrin appeared in higher amounts in membranes following several cycles of thawing and freezing. Both forms of nectadrin were recognized in Western blot analysis by mAb 79 to nectadrin (Fig. 3 A, lanes 5 and 7) and by mAbs J11d (Fig. 3 A, lanes 2 and 9), M1/69 (Fig. 3 A, lanes 3 and 8), and B2A2 (Fig. 3 A, lanes 4 and 10) to the HSA, but not by mAb 12.15 to CD2 (Fig. 3 A, lanes 1 and 6) or by polyclonal antibodies to L1 (Fig. 3 B, lane 1) or N-CAM (Fig. 3 B, lane 2). The polyclonal antibody

to nectadrin primarily reacted with h-nectadrin (Fig. 3 B, lane 3).

To further characterize the differences between the two forms of nectadrin, the glycan structures of the two forms were compared by means of lectin binding. *Datura stramonium* agglutinin, recognizing Gal β (1 \rightarrow 4)GlcNAc, strongly reacted with h-nectadrin (Fig. 3 D, lane 3). *Maackia amurensis* agglutinin, recognizing mainly NANA α (2 \rightarrow 3)Gal, reacted clearly although less well with h-nectadrin (Fig. 3 D, lane 2). Both agglutinins reacted weakly with l-nectadrin. Concanavalin A (Fig. 3 D, lane 4), *Galanthus nivalis* agglutinin (Fig. 3 D, lane 5), recognizing terminal Man-Man, and *Sambucus nigra* agglutinin (Fig. 3 D, lane 6), recognizing NANA α (2 \rightarrow 6)Gal, predominantly reacted with l-nectadrin. *Aleuria arantia* agglutinin recognizing L-Fuc α (1 \rightarrow 6)core N-acetylglucosamine (Fig. 3 D, lane 7) and *Ulex europaeus* agglutinin recognizing terminally linked fucose α (1 \rightarrow 2) (not shown) did not react with any of the nectadrin forms. Thus, the carbohydrate composition of the two forms, h-nectadrin and l-nectadrin, is better recognized by lectins that bind to terminal sugars of complex type N-linked glycans and core oligosaccharides of N-linked complex or high-mannose type glycans, respectively. The observation that the two forms of nectadrin comigrate in Western blots after N-deglycosylation (Fig. 3 E) also supports the notion that they primarily differ in the composition or number of their N-linked glycans, although small differences in the apparent molecular weights of the N-deglycosylated proteins would not be detected by this analysis.

L-nectadrin is reminiscent of the 23-kD form of the HSA described by Kay et al. (1990) in HSA-transfected COS cells as a putative precursor of the mature, higher molecular weight glycoprotein. To investigate the possible precursor-mature protein relationship between l-nectadrin and h-nectadrin, we examined the biosynthesis of nectadrin. Nectadrin was immunoprecipitated from detergent-solubilized ESb 289 cells after biosynthetically labeling the cells for 24 h with a mixture of radioactive fucose, glucosamine, and galactose. This labeling procedure was used since nectadrin contains neither cysteine nor methionine (Wenger et al., 1991) and labeling with radioactive amino acid mixtures yields only weak incorporation of radioactivity into the proteins. After this treatment, h-nectadrin was easily observed whereas l-nectadrin could not be detected (Fig. 3 C). l-nectadrin thus appears to represent a form of the antigen with a truncated carbohydrate content that is probably generated during the storage and purification process rather than being a precursor form of h-nectadrin.

Immunocytological Localization of Nectadrin at Cell-Cell Contact Sites

Our interest in nectadrin was instigated by the observation that different cell types that interact to form cell-cell contacts in tissue culture preferentially express the antigen at their contact sites (Fig. 4). This accumulation of nectadrin at cell-cell contact sites was seen by indirect immunofluorescence cytology using both monoclonal (Fig. 4) and polyclonal (not shown) antibodies to nectadrin. We examined activated splenic B-lymphocytes (Fig. 4, a and b), neuroblastoma N2A cells (Fig. 4, c and d), and cerebellar cells (Fig. 4, e-h) in culture. In cerebellar cells, 16 h after plating, nectadrin was only found on cells with a neuronal morphol-

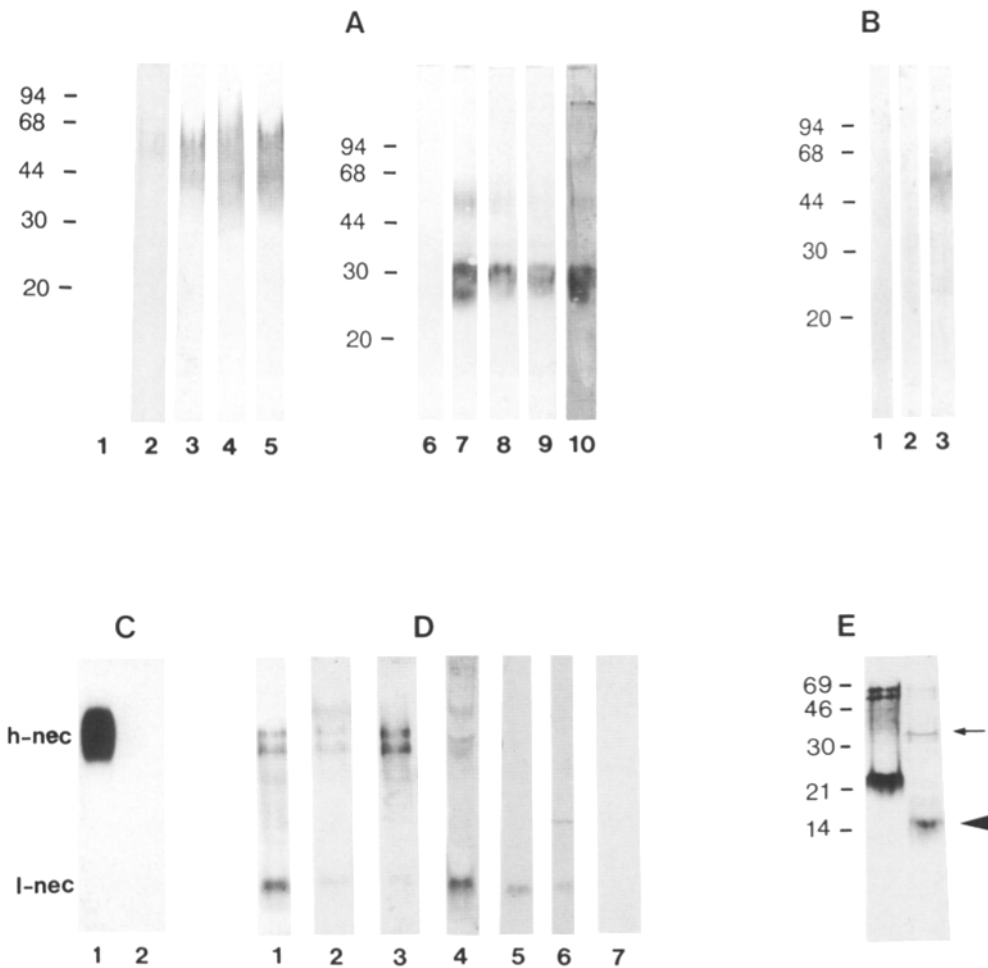


Figure 3. Comparison between h- and l-nectadrin by SDS-PAGE and Western blot analysis. (A) The biochemically purified high molecular weight form of nectadrin, h-nectadrin (lanes 1–5), and immunoaffinity-purified nectadrin, primarily containing the low molecular weight form, l-nectadrin (lanes 6–10) were separated by 8% SDS-PAGE and examined by Western blot analysis using mAbs to CD2 (12.15, lanes 1 and 6), the HSA (J11d, lanes 2 and 9; M1/69, lanes 3 and 8; B2A2, lanes 4 and 10), and nectadrin (79, lanes 5 and 7). (B) The two forms of nectadrin shown in lanes 1–5 and 6–9 in A were mixed together at a 1:1 weight ratio, separated by 8% SDS-PAGE and analyzed by Western blot analysis using polyclonal antibodies to L1 (lane 1), N-CAM (lane 2), or nectadrin (lane 3). (C) ESb 289 cells were labeled for 24 h with ^3H -labeled fucose, glucosamine, and galactose. They then were lysed in the presence of detergent. Nectadrin was immunoprecipitated using a mixture of mAbs (lane 1) or the secondary antibody to rat immunoglobulins alone

(lane 2), separated by 8% SDS-PAGE, and examined by autoradiography. (D) h- and l-nectadrin were mixed together, separated by 10% SDS-PAGE, and examined by Western blot analysis using mAb 79 (lane 1) or lectin binding analysis using digoxigenin-labeled agglutinins from *Maackia amurensis* (lane 2), *Datura stramonium* (lane 3), *Galanthus nivalis* (lane 5), *Sambucus nigra* (lane 6), and *Aleuria aurantia* (lane 7) as well as concanavalin A (lane 4). (E) h- and l-nectadrin were mixed together, incubated overnight in the absence (lane 1) or presence (lane 2) of endoglycosidase F/N-glycopeptidase F, separated by 15% SDS-PAGE, and examined by Western blot analysis using monoclonal nectadrin antibody 79. After endoglycosidase F/N-glycopeptidase F digestion, only one immunoreactive nectadrin band is seen (arrowhead). Notice that the enzyme endoglycosidase F/N-glycopeptidase F is also seen (arrow). The positions of molecular weight markers are indicated in kD at the left margins of the blots in A, B, and E. The positions of h- and l-nectadrin in C and D are marked on the left margin of C.

ogy. It was predominantly expressed at contact sites between cell bodies, between neurites and cell bodies, and between neurites. After 3 d in culture, the expression of nectadrin also was mainly confined to contact sites between neuronal cell bodies and to fasciculating neurites (Figs. 4, g and h). For comparison, antibodies recognizing all forms of N-CAM evenly bind all over the surface of N2A cells and cerebellar neurones after 1 d in culture, but preferentially label cell–cell contact sites after longer periods of culture, when the cells primarily express N-CAM₁₈₀, the largest form of N-CAM that is specifically localized at cell–cell contact sites (Pollerberg et al., 1985). After 3 d in culture under the conditions used in the present work these cells preferentially express N-CAM at their contact sites, but also elsewhere on the cell surface. Polyclonal antibodies to mouse liver membranes and to ESb 289 cell membranes have also been used and uniformly labeled the surface of the neural and spleen cells, respectively (not shown). The apparent localization of nec-

tadrin to cell–cell contact sites prompted us to examine whether nectadrin may be involved in mediating cell adhesion.

Nectadrin-dependent Aggregation of B-Lymphocytes

To evaluate whether nectadrin may mediate cell–cell adhesion, cell aggregation tests were performed. The aggregation of LPS-activated B-lymphocytes that express nectadrin was examined in the presence and absence of antibodies to nectadrin or the purified antigen itself (Fig. 5). In the presence of physiological levels of Ca^{2+} and Mg^{2+} , the cells aggregated well as evaluated by Coulter Counter analysis after 2 h of incubation in suspension at 37°C with the modal particle size measured in the Coulter Counter reaching channel 9 (Fig. 5 A). When monoclonal nectadrin antibody 79 was added in the presence of Ca^{2+} and Mg^{2+} , the aggregation of B-lymphocytes was reduced by ~70% as compared with

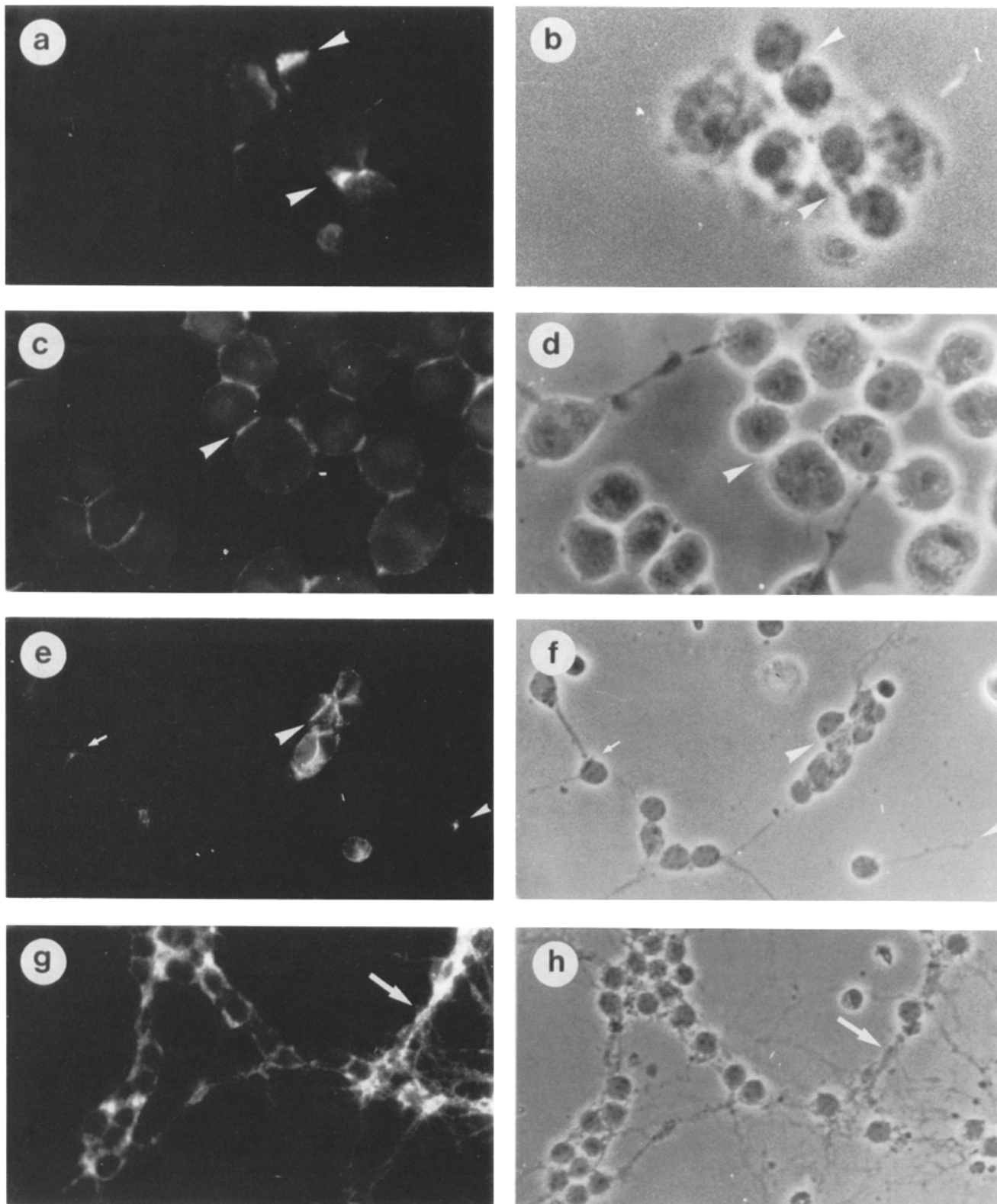


Figure 4. Indirect immunocytochemical localization of nectadrin at cell-cell contact sites in lymphoid and neural cells in culture. Primary cultures of splenocytes (*a* and *b*), N2A neuroblastoma cells (*c* and *d*), and primary cultures of early postnatal cerebellar cells (*e-h*) were grown on poly-L-lysine-coated coverslips for 2 (*a* and *b*) or 3 (*c*, *d*, *g*, and *h*) d, or for 16 h (*e* and *f*). Nectadrin was then visualized by indirect immunofluorescence staining using mAb 79 to nectadrin (*a*, *c*, *e*, and *g*); *b*, *d*, *f*, and *h* are the corresponding phase-contrast micrographs to fluorescence images *a*, *c*, *e*, and *g*, respectively. Nectadrin is concentrated at contact sites between cell bodies (*large arrowheads* in *a-f*), between neurites and cell bodies (*small arrows* in *e* and *f*), between neurites that meet head-on (*small arrowheads* in *e* and *f*), and between fasciculating neurites (*large arrows* in *g* and *h*).

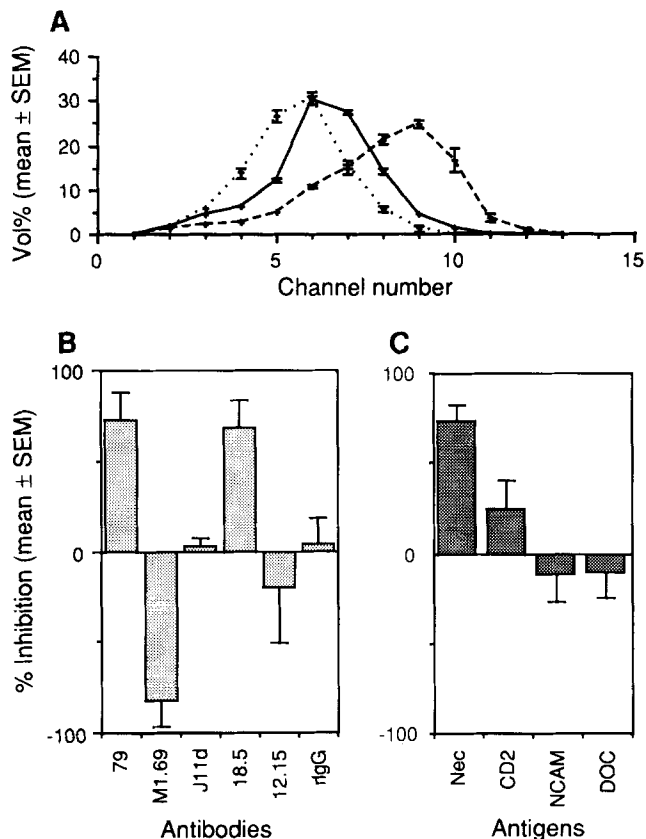


Figure 5. Determination of the aggregation of nectadrin-positive B-lymphocytes. LPS-activated B-lymphocytes were allowed to aggregate for 2 h in the absence or presence of antibodies (A and B) or purified antigens (C). The aggregation was quantitatively determined by Coulter Counter analysis. (A) Particle size distribution of the cells, indicated by vol%, before aggregation (dotted line) and after they were allowed to aggregate in the absence (broken line) or presence (solid line) of mAb 79 to nectadrin. Channel numbers 4–6, 7–9, and 10–13 correspond to single cells and small and large aggregates of cells, respectively. Values are means from one representative experiment performed in hexaplicate. (B) Effects of mAbs to nectadrin (79, M1/69, J11d), LFA-1 (18.5), and CD2 (12.15), and of polyclonal IgG antibodies from nonimmunized rats (rlgG) on the aggregation of B-lymphocytes. Values are means from at least four independent experiments, each performed in triplicate or hexaplicate. (C) Inhibition of aggregation of B-lymphocytes by the purified antigens nectadrin (Nec; a mixture of h- and l-nectadrin), CD2, or N-CAM (NCAM). The deoxycholic acid-containing buffer (DOC), in which nectadrin and N-CAM had been prepared, was used as control. Values are means from three independent experiments, each performed in hexaplicate.

their aggregation in the absence of the antibody (Fig. 5, A and B). A similar inhibitory effect was observed when purified nectadrin was added to the cells instead of the antibody (Fig. 5 C).

mAb FD18.5 to LFA-1 was also tested and found to inhibit the aggregation by ~60–70% (Figs. 5 B and 6 A). mAb J11d (Fig. 5 B) that also recognizes nectadrin as well as mAb 12.15 to CD2 (Fig. 5 B) that binds to B-lymphocytes (Altevogt et al., 1989b), nonspecific rat IgG molecules (Fig. 5 B) that include the same isotypes as the monoclonal nectadrin antibodies used and that may bind to Fc receptors, and poly-

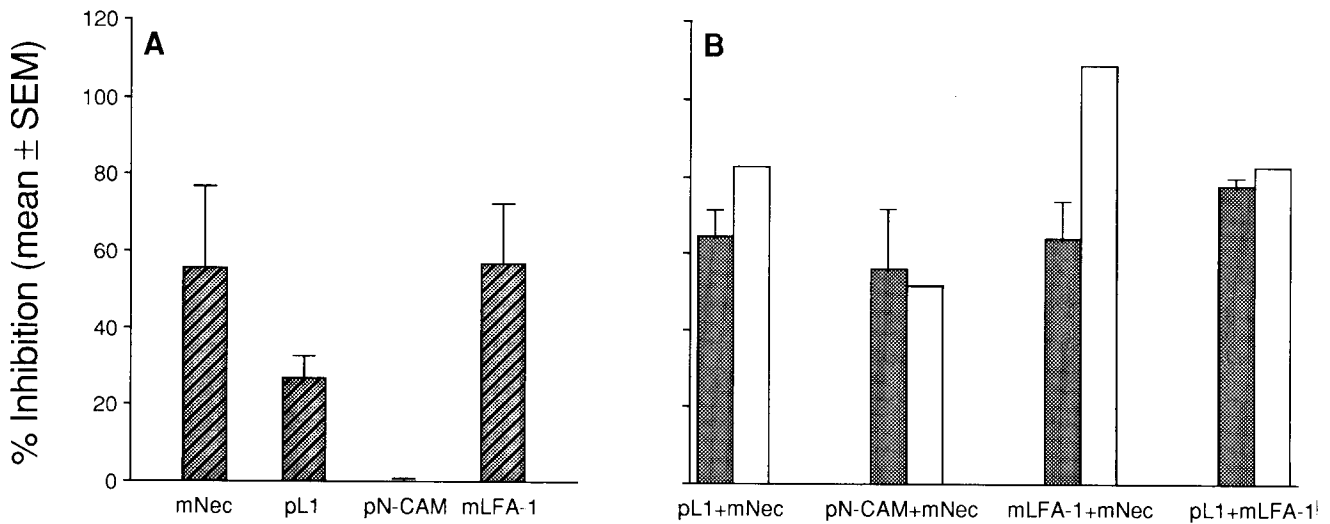
clonal antibodies to N-CAM (Fig. 6 A) that may bind to Fc receptors recognizing rabbit IgG molecules, did not significantly affect the aggregation of B-lymphocytes. Recombinant CD2 fusion protein and purified soluble N-CAM also did not significantly inhibit the aggregation of these cells (Fig. 5 C). In contrast to these results, mAb M1/69 that recognizes nectadrin (Fig. 5 B) and the polyclonal antibody to nectadrin (not shown) enhanced the aggregation of the cells. This enhancement of aggregation was observed even at antibody concentrations of up to 10 mg/ml (not shown).

Since antibodies to LFA-1 (Fig. 5 B) and L1 (Kowitz et al., 1992) have been shown to inhibit the aggregation of LPS-activated B-lymphoblasts the question arose, whether LFA-1, L1, and nectadrin have an additive effect in mediating B-lymphoblast aggregation or may display interdependent behavior. When polyclonal antibodies to L1 were added to the cells in mixture with monoclonal nectadrin antibody 79 or with monoclonal LFA-1 antibody FD18.5, the aggregation-inhibiting effects of the combined antibodies were additive (Fig. 6 B), suggesting that L1 mediates the aggregation of B-lymphoblasts by a mechanism that is independent of nectadrin and LFA-1. On the other hand, when nectadrin antibody 79 and LFA-1 antibody FD18.5, each of which inhibited B-lymphoblast aggregation by ~60% (Fig. 6 A) were mixed together cell aggregation also was inhibited by ~60% (Fig. 6 B). The combined aggregation-inhibiting effect of these antibodies to nectadrin and LFA-1 therefore is not additive, suggesting that the adhesion mechanisms mediated by nectadrin and LFA-1 may be related to one another.

The cells also aggregated in the absence of the divalent cations although much less extensively than in their presence (not shown), with the model particle size measured in the Coulter Counter reaching only channel 7 (for the channel distribution of the cells before and after aggregation in the presence of divalent cations, see Fig. 5 A). However, after 1 h of incubation in the absence of Ca^{2+} and Mg^{2+} substantial cell death was noted. In the absence of Ca^{2+} and Mg^{2+} , monoclonal nectadrin antibody 79 inhibited the weak aggregation of the cells by ~60% and almost complete inhibition was achieved by the combination of mAb 79 and polyclonal L1 antibodies (not shown). Under these conditions, the aggregation of LPS-activated B-lymphoblasts was not affected by monoclonal LFA-1 antibody FD18.5 (not shown). However, in the absence of the divalent cations many cells appeared unhealthy and a detailed analysis of their aggregation therefore was not performed.

Aggregation of Nectadrin-coated Latex Beads

Latex beads were coated either with biochemically purified h-nectadrin (h-nectadrin beads) or with immunoaffinity purified l-nectadrin containing a minor amount of h-nectadrin (l-nectadrin beads) and their ability to aggregate was then examined. By cytofluorescence analysis using constant apparatus settings, h-nectadrin and l-nectadrin beads carried similar amounts of nectadrin (relative fluorescence per bead = 154 and 97, respectively; background fluorescence values have been subtracted) to the level of nectadrin on LPS-activated B-lymphoblasts (mean relative fluorescence per cell = 151). The mean diameters of the cells and beads were 12 and 7 μ m, respectively, suggesting that nectadrin was two to three times more concentrated on the surface of the beads than on



Antibodies

Figure 6. Determination of the aggregation of B-lymphocytes in the presence of antibodies to nectadrin, L1, N-CAM, and LFA-1. LPS-activated B-lymphocytes were allowed to aggregate for 2 h in the presence or absence of monoclonal nectadrin antibody 79, monoclonal LFA-1 antibody FD18.5 (18.5), or polyclonal antibodies to L1 (*pL1*) or N-CAM (*pN-CAM*). Aggregation was quantitated by Coulter Counter analysis and the reduction in aggregation in the presence of antibodies as compared with the aggregation in their absence (% inhibition) was evaluated. The antibodies were added either separately (*A*; □ in *B*) or in different combinations (■ in *B*) as indicated under the bars in *B*. The inhibition observed in the presence of antibody combinations (▣ in *B*) was compared to the calculated sum of the mean inhibition values obtained when the corresponding antibodies were separately added to the cells (□ in *B*; values taken from *A*). Values are means from three independent experiments, each performed in triplicate.

the cells. However, in the absence of information on the membrane distribution of nectadrin in single suspended cells, the effective concentration of nectadrin on the cell surface could not be determined.

H-nectadrin beads could aggregate well. Their aggregation was observed already within 10 min (not shown). It was documented microscopically after 1 h (Fig. 7 *A,a*) and quantitated by Coulter Counter analysis after 3 h (Figs. 7, *B* and *C*, and 8 *A*) of aggregation. L-nectadrin beads formed only loose, but characteristic aggregates (Fig. 7 *A,b*) that could not be measured by Coulter Counter analysis (Figs. 7 *B* and 8 *A*), since they disintegrated upon passing through the nozzle of the apparatus due to the shearing forces. In this analysis l-nectadrin beads therefore show only few, small aggregates. Latex beads coated with L1 and with the MAG were used as positive and negative controls, respectively. L1 beads aggregated strongly (Fig. 7, *A,c* and *B*) and MAG beads did not aggregate (Fig. 7, *A,d* and *B*; see also Kadmon et al., 1990a).

The aggregation of h-nectadrin beads was completely inhibited in the presence of polyclonal antibodies to nectadrin or purified h-nectadrin as evaluated by Coulter Counter analysis after 3 h of incubation (Fig. 7 *C*). Their aggregation was inhibited by ~75% when purified l-nectadrin was added (Fig. 7 *C*), but it was perturbed neither by purified N-CAM nor by purified MAG (Fig. 7 *C*).

The observation that the aggregation of h-nectadrin beads can be inhibited by l-nectadrin suggested that the two forms of nectadrin may interact with one another. This possibility was tested by examining whether l-nectadrin beads, which do not aggregate on their own in a manner that is detectable by Coulter Counter analysis (Figs. 7 *B* and 8 *A*), can coaggregate together with h-nectadrin beads (Fig. 8, *B* and *C*).

When l-nectadrin beads and h-nectadrin beads were mixed and allowed to aggregate together, a significant decrease in the partial volume occupied by single beads as well as an increase in the partial volume occupied by small and medium-size aggregates were measured, as compared with the respective values when the beads had been mixed together only after their separate aggregation (Fig. 8 *B*). These results show that beads coated with the two forms of nectadrin can coaggregate, but that the resulting coaggregates are smaller than those produced by h-nectadrin beads alone (Fig. 8, *A* and *B*).

Discussion

In the present study we show that both purified nectadrin and the mAb to nectadrin inhibited the homotypic aggregation of B-lymphocytes by ~70%, suggesting that nectadrin may act as a ligand mediating adhesion among nectadrin-positive cells.

Nectadrin was isolated and characterized by means of the novel mAb 79 and several lines of evidence suggested that it is identical to the HSA (Springer et al., 1978). These included the serological identity of nectadrin and the HSA as well as their identical electrophoretic migration and developmentally regulated tissue distribution (M. Eckert, unpublished observations). Nectadrin and the HSA are sensitive to phospholipase C treatment in the same cell types and antibodies to the HSA partially inhibit the binding of mAb 79 to live cells (M. Eckert, unpublished observations). Furthermore, mAbs M1/69, B2A2, J11d, and 79 and the polyclonal nectadrin antibody all recognize the bacterial fusion protein encoded by the cDNA of the HSA (Wenger et al., 1991). This indicates that all these antibodies react with the 30-amino

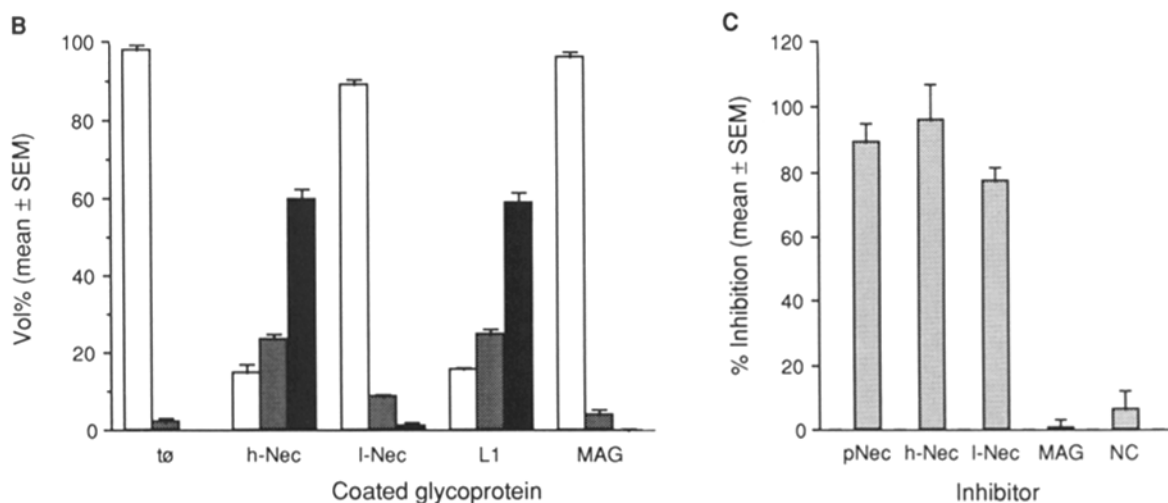
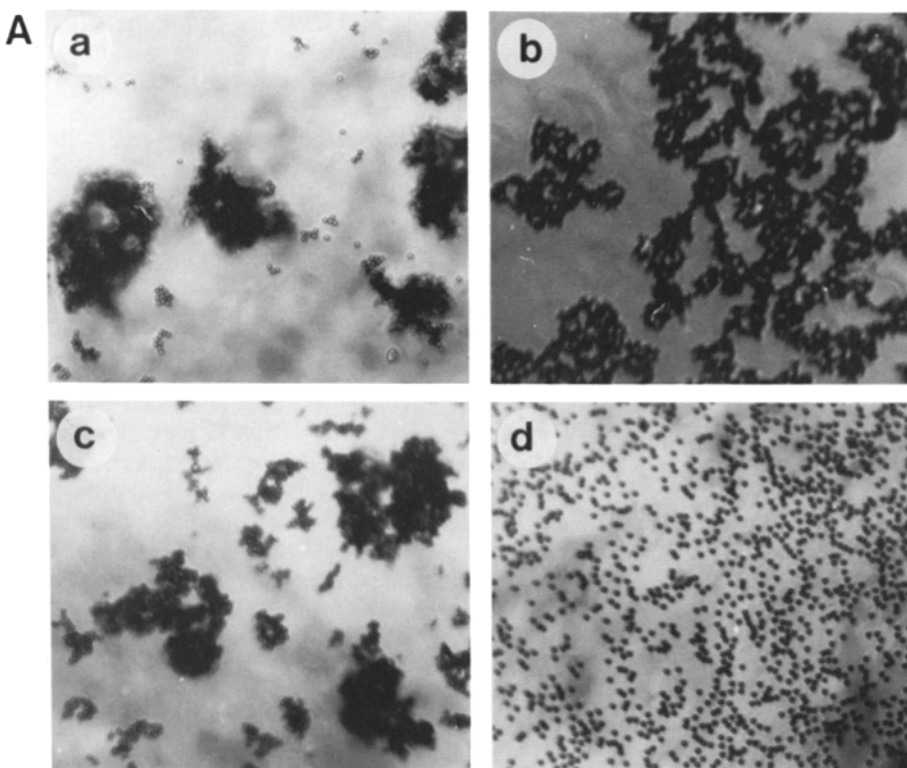


Figure 7. Determination of the aggregation of latex beads coated with nectadrin. Latex beads coated with the biochemically purified, high (h-nectadrin) or immunoaffinity purified, low (l-nectadrin) molecular weight forms of nectadrin were allowed to aggregate for 1 (A) or 3 (B and C) h. (A) The aggregation of h- (a) and l-nectadrin (b) beads was examined by phase-contrast microscopy. For controls, beads coated with L1 (c) that aggregate and beads coated with the myelin associated glycoprotein (d) that do not aggregate (Kadmon et al., 1990a) were also tested. (B) Aggregation of beads coated with h-nectadrin (h-Nec), l-nectadrin (l-Nec), L1, or MAG as measured by the Coulter Counter distribution of particle size before (t0) or after aggregation. Open, shaded, and closed bars correspond to the partial volumes (Vol %) occupied by single beads (channel numbers ≤ 6) and small (channel numbers 7, 8) and large (channel numbers ≥ 9) aggregates, respectively. (C) Beads coated with h-nectadrin were allowed to aggregate for 3 h in the absence or presence of purified h-nectadrin (h-Nec), l-nectadrin (l-Nec), MAG, or N-CAM (NC), or polyclonal antibodies to nectadrin (pNec). Aggregation was quantitatively determined by Coulter Counter analysis. The reduction in the formation of large aggregates of h-nectadrin beads in the presence of these additives as compared to the formation of large aggregates in the absence of additives is indicated as percent inhibition. Values in B and C are means from at least three independent experiments, each performed in triplicate or hexuplicate.

acid polypeptide core of the glycoprotein (Kay et al., 1990; Wenger et al., 1991).

Although nectadrin has different apparent molecular masses in different tissues and cell lines, only one nectadrin

gene, designated gene A, is expressed in all the tissues that have been examined, brain, thymus, bone marrow, and spleen as well as in pre-B and erythroid cell lines (Rougon et al., 1991; Wenger et al., 1991), and in the T-lymphoma

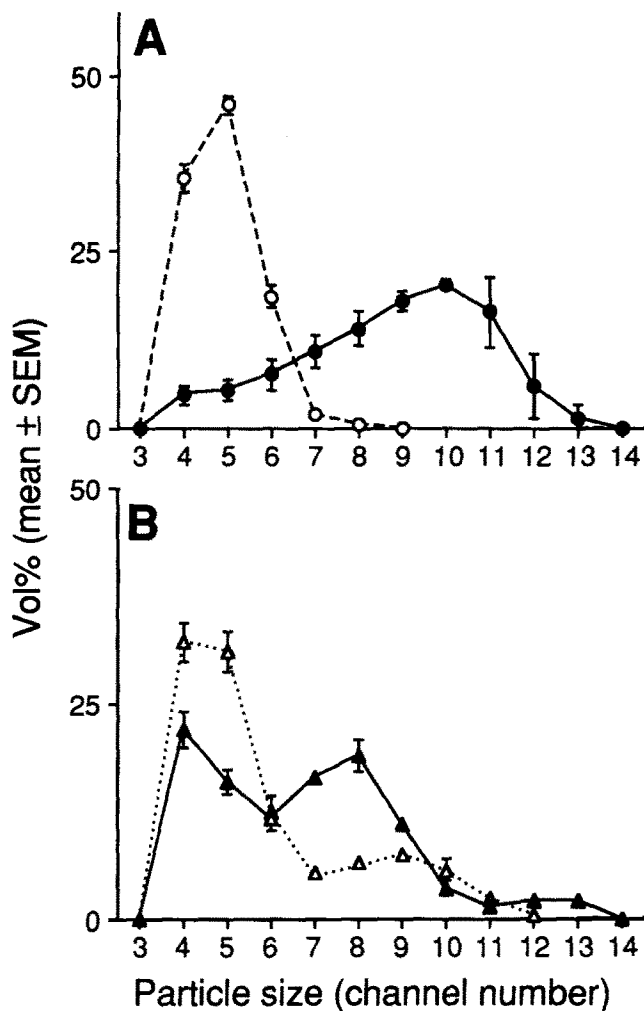


Figure 8. Determination of the coaggregation of l-nectadrin-coated beads with h-nectadrin-coated beads. Latex beads coated with l-nectadrin or h-nectadrin were allowed to aggregate for 3 h either separately or mixed together. Aggregation was quantitated by Coulter Counter analysis. (A) Distribution of particle size of l-nectadrin (---○---) and h-nectadrin (—●—) beads after separate aggregation. Single beads, small and large aggregates are measured in channel numbers ≤ 6 , 7–8, and ≥ 9 , respectively. Whereas l-nectadrin beads only form few, small aggregates by this method, h-nectadrin beads aggregate to form large aggregates. Values are from one representative experiment performed in hexaplicate. (B) L-nectadrin and h-nectadrin beads were mixed together either before (—▲—) or after (···△···) aggregation. Coaggregation of the differently coated beads is indicated by a reduction in the partial volume occupied by single beads (channel numbers 4–6) and an increase in the partial volume occupied by aggregates (channel numbers 7–14). L-nectadrin beads that do not aggregate on their own, when measured by Coulter Counter analysis, aggregate together with h-nectadrin beads to form small and medium sized aggregates. Values are means from one representative experiment performed in hexaplicate.

cell line ESb 289 (P. Nielsen, personal communication). There has been no evidence for alternative splicing of this nectadrin gene A (Kay et al., 1990; Wenger et al., 1991). The mature polypeptide coded by gene A is expected to have only 30 amino acids with a molecular mass of 2.7 kD (Kay et al., 1990; Wenger et al., 1991) and the differences of up to 40 kD in the apparent molecular mass of nectadrin in

different cell types appear to result mainly, if not only from variations in its N-linked and O-linked glycosylation (Alterman et al., 1990; Kay et al., 1990; Rougon et al., 1991) that accounts for 85–95% of its apparent molecular mass.

In the present study, two forms of nectadrin of 23–30 and 40–60 kD that were both recognized by Western blot analysis by the different nectadrin antibodies were differentially isolated from ESb 289 cells. Since ESb 289 cells only express the phosphatidylinositol-anchored gene A product of nectadrin these different forms probably differ from one another in their posttranslational modifications, presumably in the content and/or structure of their glycans. This notion is supported by the similar migration of h-nectadrin and l-nectadrin in SDS-PAGE after *N*-deglycosylation and by differences in the binding of lectins to the two forms of the antigen. L-nectadrin appeared in ESb 289 cell membrane preparations in increasing amounts, relative to h-nectadrin, after long storage and handling of the membranes, indicating that it had been generated from h-nectadrin during this process. Biosynthetic labeling experiments subsequently failed to demonstrate the presence of l-nectadrin in live ESb 289 cells. The low molecular mass form of nectadrin therefore appears to be a degraded form of h-nectadrin that primarily differs from h-nectadrin in its carbohydrate content.

These two forms of nectadrin not only differ in their structure, but also in their function in self-recognition. As evaluated from the aggregation of latex beads coated with the different forms of nectadrin, the adhesive properties of the two forms were strikingly different in that the 40–60-kD form appeared to be much more adhesive than the 23–30-kD form. Interestingly, the two forms can interact with one another, indicating that the binding between nectadrin molecules may possibly involve different domains that are in part common to both forms of the molecule. These observations suggest that nectadrin may mediate cell adhesion by a nectadrin-nectadrin interaction that depends on its structure, most likely on its oligosaccharide composition. Moreover, in view of the unusually high carbohydrate content in the nectadrin molecule, the glycan structures of nectadrin are likely to have direct functional importance and it is tempting to speculate that the binding between nectadrin molecules may even result from glycan-glycan interactions. A specific glycan-glycan interaction that mediates cell adhesion has previously been reported for the Lewis \times (Le^x) determinant. Le^x glycans have been shown to mediate the adhesion between F9 embryonal carcinoma cells by homophilic interaction (Eggens et al., 1989) and have been suggested to have a role in the compaction of the murine embryo at the morula stage (Fenderson et al., 1984).

The conjecture that different domains of the nectadrin molecule may have diverse functional roles was pointed at by the variable effects that monoclonal and polyclonal nectadrin antibodies had on B-cell aggregation. In contrast to the inhibition of cell aggregation by mAb 79, other nectadrin-specific mAbs, J11d and B2A2, did not interfere with the aggregation whereas monoclonal nectadrin antibody M1/69 and the polyclonal nectadrin antibody enhanced the aggregation of the cells. This increase in aggregation occurred also when the antibodies were used at very high concentrations, thus rendering the possibility of cross-linking between the cells unlikely. The possibility of cross-linking also seems unlikely in view of the observation that polyclonal nectadrin

antibodies that stimulated cell aggregation completely inhibited the aggregation of nectadrin-coated latex beads when used at similar concentrations. No increase in aggregation was seen when polyclonal rabbit IgG antibodies to N-CAM or nonspecific rat IgG molecules that included the same isotype as mAb M1/69 were used, ruling out that the enhanced aggregation would have resulted from antibody-dependent Fc receptor-mediated stimulation of aggregation. It therefore is conceivable that antibody M1/69 and the polyclonal antibody to nectadrin may either directly enhance the capacity of nectadrin to mediate cell binding by altering the conformation of the molecule, or indirectly trigger other adhesion molecules via intracellular second messengers by binding to nectadrin.

In this context, it is interesting that the mixture of nectadrin antibody 79 and LFA-1 antibody FD18.5 inhibited the aggregation of B-lymphoblasts to the same extent as when each of these antibodies had been used on its own. This observation could indicate that the LFA-1 and nectadrin-dependent adhesion mechanisms which are perturbed by these antibodies may be related to one another. Since nectadrin appears to mediate the aggregation of cells and beads also in the absence of divalent cations, that is, under conditions that render LFA-1 inactive, it does not seem likely that the two molecules mediate cell adhesion as a ligand and receptor pair. However, in view of results showing that nectadrin (Kadmon, G., M. Eckert, P. Horstcorte, and M. Schachner, manuscript submitted for publication) and its human homologue, CD24 (Fischer et al., 1990), are involved in mediating signal transduction, it is possible that LFA-1 and nectadrin are functionally related via a second messenger pathway. Similar cellular consequences of antibody binding to cell-surface antigens have previously been reported to activate LFA-1-dependent T-cell adhesion: LFA-1-dependent homotypic T-cell adhesion is transiently enhanced after binding of CD3 antibodies and persistently enhanced after binding of CD2 antibodies to the cells (van Kooyk et al., 1989). The stimulation of LFA-1 after the binding of antibodies to CD3 and CD2 appears to be regulated by a protein kinase C-dependent mechanism (van Kooyk et al., 1989). In T-cells LFA-1 is also activated via protein kinase C by some CD44 antibodies (Koopman et al., 1990), whereas another CD44 antibody has been reported to inhibit the CD2/LFA-3-mediated adhesion between T-cells and erythrocytes, possibly via a *cis* interaction between CD44 and CD2 on the cell membrane (Hale et al., 1989).

Nectadrin is expressed in polymorphic forms by as different cells as neurones, lymphocytes, monocytes, and erythrocytes and it is conceivable that it may mediate cell adhesion by a variety of mechanisms. In addition to its putative ability to mediate cell adhesion by a nectadrin-nectadrin interaction, it is possible that it may also bind to glycan-binding adhesion molecules. Several lectins and glycosyl transferases that bind to carbohydrates of different specificities have been suggested to mediate neuronal adhesion in the development of the nervous system, and LECAMs, adhesion molecules that carry an NH₂-terminal lectin domain, serve as adhesion ligands among cells of haematopoietic lineage and endothelial cells (for reviews, see Olden et al., 1982; Rutishauser and Jessell, 1988; Brandley et al., 1990; Dustin and Springer, 1991).

In conclusion, nectadrin is a novel cell adhesion molecule

that is structurally different from other known cell adhesion molecules in that it contains only a very small polypeptide core and manifests an unusual degree of glycosylation. It may mediate cell adhesion by self-binding and by other molecular mechanisms that appear to implicate glycan-glycan interactions.

For their generous gifts of the following reagents, we would like to express our gratitude to M. Simon for antibodies M1/69 and FD18.5, to M. Pierres and H. von Boehmer for antibodies J11d and B2A2, respectively, to B. Kyewski for antibodies 30-H12 and RA3-6C2, to R. Rutschmann for recombinant CD2 fusion protein, and to N. Hilschmann for ESb cell membranes. We were also thankful to A. Kowitz for her help in the cytofluorographic analysis.

This work was supported by the "Kommission zur Förderung der wissenschaftlichen Forschung."

Received for publication 20 November 1991.

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