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A simpler sort of antibody

(light chain monomer/nevus antigen/melanoma)

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ABSTRACT **The monoclonal antibody NEMO is directed against a molecule expressed by human cells of the melanocytic lineage. Although obtained by conventional immunization and fusion procedures, NEMO consists solely of κ light chain. SDS/PAGE analysis indicates that the κ chains are present as both monomers and dimers. When these two forms were separated by gel filtration, only the monomeric form bound antigen. As κ light chains from the myeloma MOPC-41 and the hybridoma MORK do not bind to the melanocytic cells, we conclude that the binding specificity of NEMO resides in the variable region.**

A standard antibody is a tetrameric structure consisting of two identical immunoglobulin heavy chains and two identical light chains. In such antibodies, the variable regions of both the light and heavy chains generally contribute to antigen specificity, although the contribution of the individual chains to specificity need not be equal. For example, the average binding constant of rabbit IgG to the hapten 2,4-dinitrophenyl was $3 \times 10^8\ M^{-1}$, while for the heavy chain dimers and the light chain it was $6 \times 10^4\ M^{-1}$ and 200–500 M^{-1}, respectively (1). Separated light or heavy chains have sometimes been found to retain some antigen-binding activity (2–4), and a cloned heavy chain variable region domain has been reported to bind antigen with good affinity (5). Bence Jones proteins, the free light chain dimers found in the urine of patients with multiple myeloma, have some antigen-binding activity (6, 7). An immunoglobulin light chain dimer with CD4 antigen specificity showed an association constant of $2.5 \times 10^9\ M^{-1}$ (8). Here we report the isolation of a monoclonal antibody that is highly specific for a molecule expressed by human cells of the melanocytic lineage and that consists only of a κ light chain; only in the monomeric form does this κ light chain specifically bind to its cognate antigen.

MATERIALS AND METHODS

Antibodies and Cells. The hybridoma MORK was established as described (9). Myeloma MOPC-41 (10) protein was kindly provided by Luciana Forni (Hoffmann–La Roche). Melanoma cell line C32-TG and myeloma P3X63Ag8.653 were obtained from the American Type Culture Collection. Hybridoma BM2H8 (IgM, κ) was kindly provided by Barbara Mayer in our laboratory.

Immunization and Cell Fusion. A female BALB/c mouse was immunized intraperitoneally with a membrane fraction (11) prepared from two specimens of common acquired nevi. *Bordetella pertussis* (10^9 cells) was used as adjuvant. Three days later, the spleen was removed, and the cells were fused with mouse myeloma P3X63Ag8.653 as described (12); interleukin 6 (Boehringer Mannheim; 300 units/ml) was added as a growth factor. Hybridoma supernatants were screened for reactivity with frozen tissue sections of melanocytic lesions. The antibody NEMO (nevus reactive monomer) was cloned by limiting dilution.

Immunohistochemistry and Flow Cytometry. Frozen tissue sections and C32-TG cell cytospins were stained as described (13) with an affinity-purified, peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dianova, Hamburg, F.R.G.) as second antibody and as substrate 3-amino-9-ethylcarbazole (Sigma; 0.25 mg/ml) and 0.003% H_2O_2 in 0.1 M acetate buffer (pH 4.9). Undiluted NEMO or MORK supernatant or purified MOPC-41 protein (20 μg/ml) was used as first antibody. For flow cytometric detection of cytoplasmic antigens, the cells were fixed in methanol for 3 min on ice, washed twice in phosphate-buffered saline (PBS) containing 0.01% sodium azide, incubated with NEMO (as supernatant or as chromatographic fractions) or MORK for 30 min at 4°C, and then incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (Dako) for 30 min at 4°C. Background fluorescence due to the accumulation of unbound fluorescein isothiocyanate into the cytoplasmic compartment was reduced by washing twice after each incubation step. Prior to each wash, the cells were left in PBS containing 0.01% azide for 10 min at room temperature. Cells were analyzed with a FACScan (Becton Dickinson).

Isotype Determination. Antibody isotypes were determined with a commercially available kit (PharMingen).

Biosynthetic Labeling and SDS/PAGE Analysis. Hybridomas BM2H8 and NEMO were incubated for 1 hr in methionine-free medium containing 5% dialyzed fetal calf serum and then cultured overnight in the same medium with [^{35}S]methionine plus [^{35}S]cysteine (ICN; 50 μCi/ml; 1 Ci = 37 GBq). The supernatants were incubated with a rabbit anti-mouse immunoglobulin antiserum (specific for both heavy and light chains) coupled to protein A-Sepharose; bound material was eluted by heating the Sepharose pellets in sample buffer for 5 min at 95°C and analyzed on a 10% SDS/PAGE gel under reducing or nonreducing conditions. The fixed gel was treated with Amplify (Amersham) and dried; x-ray film was exposed overnight.

Gel-Filtration Chromatography. NEMO culture supernatant was concentrated 30-fold, and 1 ml was loaded onto a gel-filtration AcA-44 (IBF, Villeneuve-la-Garenne, France) 2.5 × 100 cm column, which had been equilibrated with 0.05 M Tris·HCl/0.15 M NaCl, pH 7.5. Protein elution was monitored at 280 nm with a 2138 UVICORD-S spectrophotometer (LKB), and 3-ml fractions were collected. The fractions were tested for the presence of κ chain by ELISA, assayed for NEMO activity by flow cytometry, and assessed for the distribution of κ chain by Western blotting. The fractions containing albumin were discarded; those containing κ chain monomers or dimers were pooled, concentrated to 1 ml, and loaded onto a Sephacryl S-100 HR (Pharmacia LKB) 1.6 × 100 cm column equilibrated with the above-described buffer. Fractions of 3 ml were collected and analyzed as described above.

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ELISA. Chromatographic fractions were monitored for κ chain with an ELISA. Polyvinyl chloride microtiter plates were coated with a rabbit anti-mouse κ chain antiserum (Bethyl Laboratories, Montgomery, TX) for 1 hr at room temperature. The plate was then washed in PBS/0.05% Tween 20 and blocked with 0.1% gelatin in PBS. After 50 μl of each fraction was added, the coated plates were incubated for 1 hr, washed in PBS/0.05% Tween 20, and incubated with a peroxidase-conjugated, rabbit anti-mouse immunoglobulin antiserum for 1 hr. Bound κ chain was visualized with a substrate composed of *ortho*-phenylenediamine (2 mg/ml; Sigma) in 0.1 M citrate buffer (pH 5) and 0.002% H_2O_2. Color intensity was read spectrophotometrically at 450 nm (EAR 400 AT, SLT Labinstruments, Research Triangle Park, NC).

Western Blotting. Chromatographic fractions were concentrated 20-fold in a Centricon-10 miniconcentrator (Amicon); 15-μl samples were separated in SDS/12% PAGE under nonreducing conditions and electrophoretically transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell) as described (14). The membrane was blocked in 0.1% gelatin in PBS for 1 hr, incubated with a peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum for 1 hr, and then exposed to a substrate solution of 1.26 mM *o*-dianisidine hydrochloride (Sigma)/15.3 mM sodium nitroferricyanide (Sigma)/0.007% H_2O_2.

RESULTS AND DISCUSSION

A Monoclonal Antibody Specific for the Melanocytic Lineage. The hybridoma NEMO was identified by screening supernatants for reactivity with frozen tissue sections of melanocytic nevi. In 32 of 35 specimens examined, NEMO was found to bind to the nevus cells but not to normal melanocytes in the epidermis (Fig. 1). No reactivity was observed with any other structure in the skin, including epidermis, hair follicles, blood vessels, glands, and muscles. The antibody reacted with 8 out of 24 melanoma cell lines tested. As no reactivity could be detected by immunofluorescence on living, unfixed cells, the epitope detected by NEMO seems to be located only in the cytoplasm.

The Antibody Consists of κ Chain Only. NEMO reacts with anti-κ reagents but not with antisera specific for λ light chain or any of the heavy chain isotypes tested: IgM, IgG1, IgG2a, IgG3, IgA. Although the presence of δ or ε heavy chain was not formally excluded by the typing reagents used, it seemed likely that the NEMO antibody is composed solely of κ chain. The experiment shown in Fig. 2 confirms that the NEMO antibody does not contain heavy chain. The biosynthetically labeled immunoglobulin chains secreted by NEMO, along with those of a control hybridoma BM2H8 (IgM, κ), were immunoprecipitated and separated under reducing (Fig. 2*A*) and nonreducing (Fig. 2*B*) conditions in SDS/PAGE. Under reducing conditions, both μ and κ chains of BM2H8 are clearly visible at about 65 kDa and 23 kDa, respectively; the NEMO product, on the other hand, runs as a single molecular species of about 25 kDa. Under nonreducing conditions, most of the BM2H8 material is present as a high molecular mass complex at over 200 kDa; most of the NEMO antibody, on the other hand, remains at about 25 kDa, but a second band at about 50 kDa is now also seen. Identical results were obtained by Western blot analysis of immunoprecipitates and of total NEMO supernatants. The 50-kDa band must represent light chain dimers, as confirmed below. Thus, the NEMO hybridoma produces an immunoglobulin κ light chain that is present in both monomeric and dimeric forms.

Only the Monomeric Form Binds to the Specific Antigen. To determine whether the antibody activity resides in the monomer or in the dimer, NEMO supernatant was subjected to a two-step chromatographic separation. The fractions collected from the second column were tested for the presence

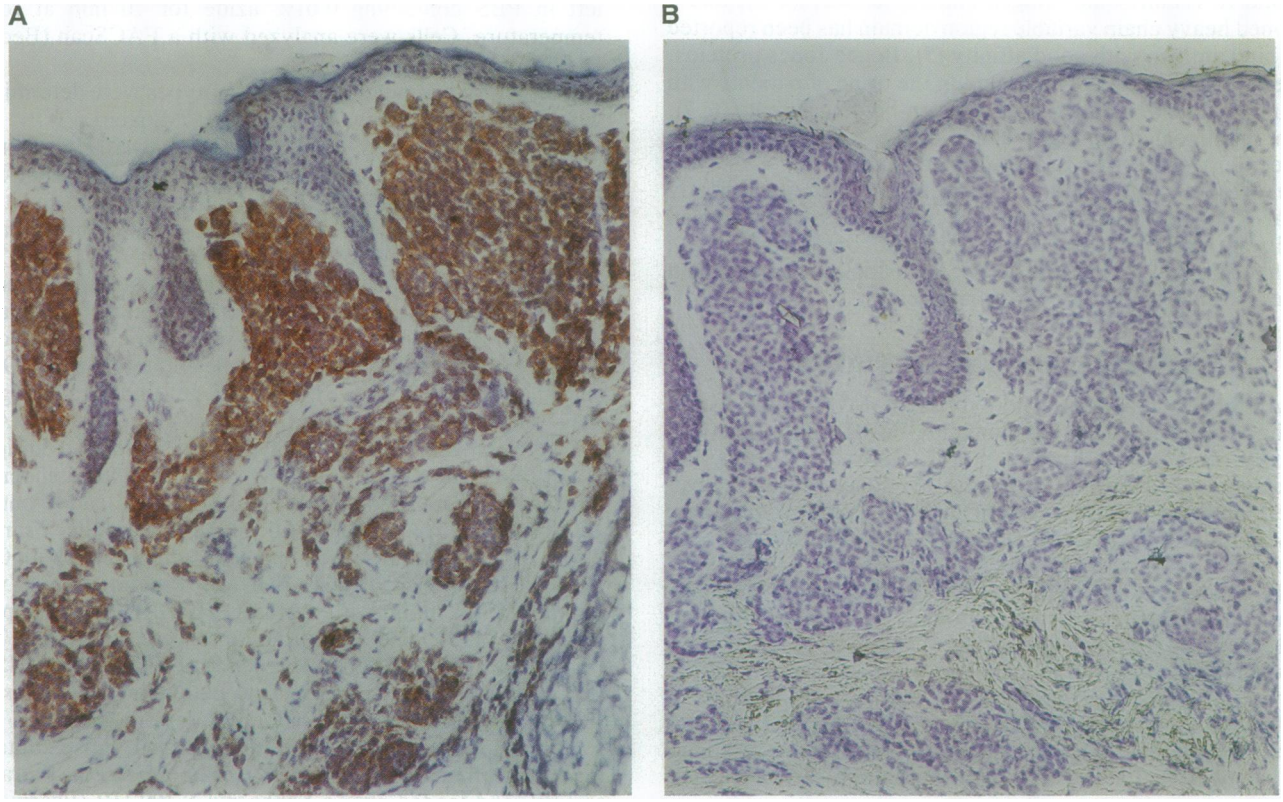

FIG. 1. Reactivity of antibody NEMO with melanocytic nevus cells. Reactivity of NEMO (*A*) and MOPC-41 (*B*) on frozen sections of a congenital nevus visualized by immunoperoxidase staining. NEMO reacts with nevus cells but not with other structures in the skin. No staining is seen with the κ chain of the myeloma MOPC-41; the same result was obtained with the κ chain of the hybridoma MORK (data not shown). (×120.)

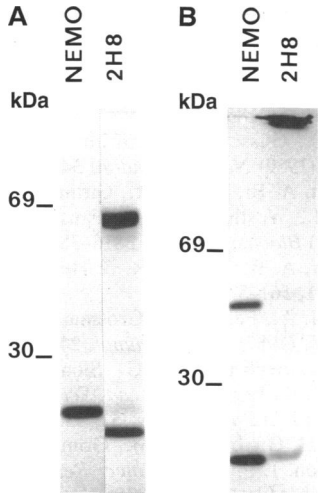

FIG. 2. SDS/PAGE analysis of NEMO immunoglobulin. Immunoprecipitates from biosynthetically labeled supernatants of NEMO and a control hybridoma, BM2H8 (IgM, κ), were separated under reducing (*A*) and nonreducing (*B*) conditions. Under reducing conditions, the NEMO antibody migrates as a single species of about 25 kDa (*A*; left lane). Heavy and light chains of control antibody BM2H8 are seen at about 65 and 23 kDa (*A*; right lane). Under nonreducing conditions, κ dimers of the NEMO antibody are seen in addition to the monomers (*B*; left lane), while for the control BM2H8, the IgM molecule is seen on the top of the lane as a high molecular weight complex (*B*, right lane). Molecular size standards are on the left of each panel.

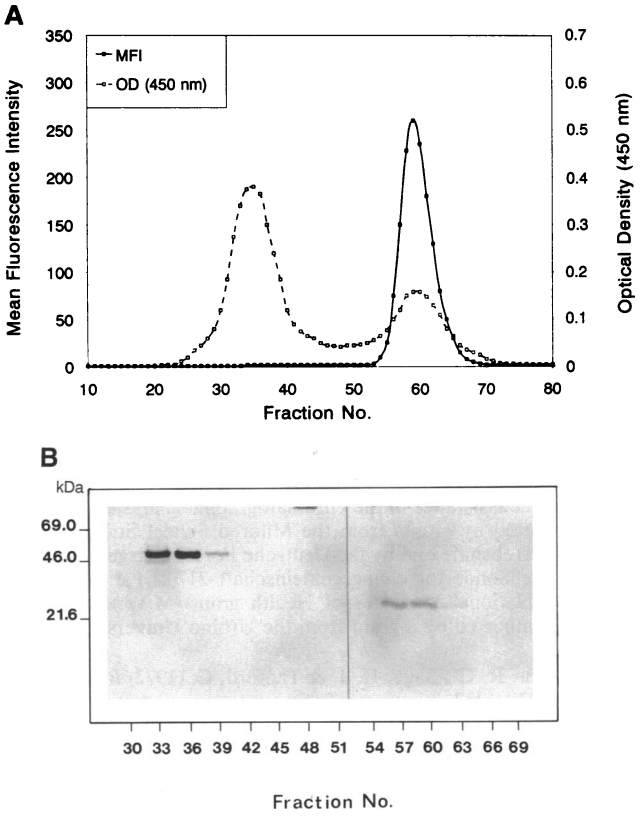

FIG. 3. Chromatographic separation of monomeric and dimeric κ chains from NEMO supernatants. After separation of the albumin fraction, monomeric and dimeric NEMO κ chains were further separated on a Sephacryl S-100 HR column, and 3-ml fractions were collected. (*A*) The dashed line indicates the presence of κ chains in each chromatographic fraction, as determined by ELISA (OD at 450 nm). The solid line is the antibody activity profile; the values are the relative mean fluorescence intensity of each fraction tested by immunofluorescence on fixed C32-TG melanoma cell line and analyzed by FACScan. (*B*) Some fractions were analyzed by Western blotting. NEMO dimeric κ chains are located in fractions 30–39, which corresponds to the first ELISA peak; NEMO monomers are in fractions 54–63, which corresponds to the second ELISA peak and to the antibody activity peak. Molecular size standards are on the left side of the blot.

of κ chain by ELISA, for the distribution of κ monomer and κ dimer by Western blotting, and for antibody activity by immunofluorescence on the C32-TG melanoma cell line. In Fig. 3*A*, the dashed line shows that there are two peaks in ELISA. One peak is located in fractions 26–40, in which ≈50-kDa proteins are eluted; the second peak is located in fractions 55–69, in which ≈25-kDa proteins are eluted. The two molecular forms are well separated, and their apparent molecular masses correspond to what would be expected for the dimer and monomer of the NEMO κ chain. To confirm this hypothesis, fractions 33, 36, and 39 from the first ELISA peak were concentrated, separated on SDS/PAGE, and blotted; as shown in Fig. 3*B*, these fractions contain only the NEMO κ chain dimer represented by the band with an apparent molecular mass of ≈50 kDa. Fractions 57, 60, and 63 from the second immunoglobulin peak contain the κ chain monomer of 25 kDa.

The solid line in Fig. 3*A* represents the binding of NEMO antibody to C32-TG (mean fluorescence intensity of each fraction). No antibody activity is found in fractions 30–40, which contain the dimer. The peak of antibody (i.e., binding) activity, which is observed in fractions 54–69, overlaps with the second immunoglobulin peak shown to contain the monomer. Identical results were obtained when the column fractions were tested by immunoperoxidase staining of C32-TG cytospins. Since antibody binding activity is only detectable in this region, and since the NEMO monomer is the only molecular species in this peak recognized by the peroxidase-conjugated second antibody (as seen in Western blot analysis), we conclude that the NEMO antibody activity resides solely in the monomer. Thus, the antigen-binding site seems to be blocked or altered by dimer formation.

Binding Activity Resides in the Variable Region. To qualify for consideration as a bona fide antibody, NEMO must bind to its specific ligand in the melanocyte via its variable region. Indeed, free κ chains from myeloma MOPC-41 and from hybridoma MORK do not bind to nevus or melanoma cells, even though they are both well recognized by the second antibody, and even though they are both present as dimers and monomers. Thus, the antigen-binding activity of NEMO appears to be mediated by the variable region of the κ chain.

The alternative would be that NEMO would bind to a receptor for light chains. If NEMO bound to a light chain receptor, it might be expected that NEMO would bind to a variety of tissues. In fact, NEMO binds only to cells of the melanocytic lineage that are found in melanocytic nevi and some melanomas; there was no binding to several carcinomas tested or to any structure present on the skin, stomach, liver, colon, kidney, or breast tissue (data not shown).

Although the nature of the molecule recognized by NEMO has not yet been elucidated, both of the above observations suggest that NEMO functions, not as a ligand for a receptor, but as an antibody binding to its specific antigen.

Concluding Remarks. Since light chains alone can bind antigen, the possibility is raised that the binding specificity of the free light chain is an important factor in the onset of the clinical disorder known as AL type amyloidosis, which is characterized by the deposition of κ light chains in various organs (15).

Primarily because of their small size, single-chain antibodies (16) are currently in great demand for a variety of therapeutic applications. We have described here a highly specific anti-

body consisting only of κ chain, the only single chain antibody found among 107 hybridomas generated by the same immunization and screening procedure. Since light chain alone cannot be displayed on the membrane, the cells that produce such an antibody are probably derived by heavy chain loss from cells that originally produced a standard antibody consisting of both heavy and light chains. Because right after fusion hybridomas tend to lose many chromosomes, it seems likely that there are numerous other examples of monomeric light chain antibodies among the hundreds of thousands of hybridomas generated since the fusion method was described (12). Thus it might be feasible to use conventional techniques to generate naturally occurring monomeric light chain antibodies for many antigenic specificities.

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