Normal human serum contains a natural IgM antibody cytotoxic for human neuroblastoma cells

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ABSTRACT Neuroblastoma (NB) is characterized by the second highest spontaneous regression of any human malignant disorder, a phenomenon that remains to be elucidated. In this study, a survey of 94 normal human adult sera revealed a considerable natural humoral cytotoxicity against human NB cell lines in approximately one-third of the tested sera of both genders. Specific cell killing by these sera was in the range of 40% to 95%. Serum cytotoxicity was dependent on an intact classical pathway of complement. By several lines of evidence, IgM antibodies were identified as the cytotoxic factor in the sera. Further analyses revealed that a 260-kDa protein was recognized by natural IgM of cytotoxic sera in Western blots of NB cell extracts. The antigen was expressed on the surface of seven human NB cell lines but not on human melanoma or other control tumor cell lines derived from kidney, pancreas, colon, bone, skeletal muscle, lymphatic system, and bone marrow. Furthermore, no reactivity was observed with normal human fibroblasts, melanocytes, and epidermal keratinocytes. The antigen was expressed in vivo as detected by immunohistochemistry in both the tumor of a NB patient and NB tumors established in nude rats from human NB cell lines. Most interestingly, the IgM anti-NB antibody was absent from the sera of 11 human NB patients with active disease. The anti-NB IgM also could not be detected in tumor tissue obtained from a NB patient. Collectively, our data suggest the existence of a natural humoral immunological tumor defense mechanism, which could account for the in vivo phenomenon of spontaneous NB tumor regression.

Neuroblastoma (NB) is the most common extracranial solid neoplasm seen in infancy (1). However, despite improved diagnostic and therapeutic modalities, NB is still associated with very poor prognosis (1, 2). Approximately 15% of all childhood cancer deaths are caused by NB (1). Interestingly, however, this neuroectodermal tumor is characterized by the second highest spontaneous regression rate of human malignant disorder (1, 3). While some authors suggest that growth-controlling or apoptotic mechanisms are responsible for the observed phenomena (1, 4, 5), others favor immunological factors such as the action of natural killer cells or specific antibodies that lead to tumor regression (1, 6–8). Recently, the existence of natural IgM antibodies cytotoxic for human NB cells was reported in gestational sera during the second and third trimester (9).

In this study, we surveyed normal human serum (NHS) of 94 healthy adult individuals of both genders for cytotoxicity against NB cell lines. Approximately one-third of all tested sera exhibited considerable cytotoxicity (40% to 95% cell killing) that was mediated by natural IgM and the classical pathway of complement. The natural IgM recognized a 260-kDa antigen on human NB cell lines. The antigen was also found to be expressed in vivo in a tumor of a NB patient and in human NB tumors grown in nude rats. Most interestingly, the analysis of sera from NB patients with active disease revealed the absence of the cytotoxic activity against NB, suggesting an important role of natural IgM antibodies in the defense against human NB.

MATERIALS AND METHODS

Human Cell Lines. Human NB cell lines were obtained from R. C. Seeger (LA-N-1, LA-N-5; University of California, Los Angeles) (10), N.-K. V. Cheung (NMB-7; Memorial Sloan-Kettering Cancer Center, New York) (11), C. Schaller (SH-SY5Y; University of Hamburg) (12), and the American Type Culture Collection (ATCC; SK-N-SH, IMR-32, SK-N-MC). The human melanoma cell lines SK-MEL-170 and SK-MEL-93–2 have been described (13, 14). K562, CEM, TE-85, and RD cells were from ATCC, WiDr (15) and Panc Tu-I cells (16) were from H. Kalthoff (University of Kiel, Germany), and SK-RC-29 cells (17) were from L. J. Old (Memorial Sloan-Kettering Cancer Center). Normal human epidermal keratinocytes, dermal fibroblasts, and skin melanocytes were from Clonetics (San Diego) and were cultured according to the supplier’s instructions.

Human Sera and Antibodies. Human serum was obtained from 94 healthy adult volunteers (age 18–52 years) and from 11 NB patients (age 5 months–14 years) before and during chemotherapy. As an age-matched control for the NB patients, sera from 229 healthy children of different age groups (group I, age 0–1 year, n = 119; group II, age 2–6 years, n = 41; group III, age 7–14 years, n = 39) were used. Heat inactivation (56°C for 30 min), decomplexation with cobra venom factor (184 μg/ml), and MgEGTA treatment (2.5 mM) of NHS were performed as described (18). The following murine monoclonal antibodies were supplied: anti-GD2 (BW704) by K. Bosslet (Behringwerke) (19); anti-GD3 (R24) by L. J. Old (13, 14); and anti-proteoglycan (9.2.27) by A. C. Morgan (National Cancer Institute, Frederick, MD) (20). Dichlorotriacilinamino-fluoresceine-conjugated goat anti-human IgG (Fcε specificity) and anti-human IgG (heavy and light chain specific) (Dianova, Hamburg, Germany) were used in cellular binding studies (1:50 dilution). A fluorescent sulfocyanine dye (Cy3)-conjugated rabbit anti-human IgM (Fcε specificity) (Dianova) was used in immunocytochemical and -histochemical analyses (1:500 dilution). Affinity-purified Fab(α′)′ fragments against human Fcy and human Fcεμ were from Dianova. Goat antibodies against human IgM (μ-specific) and IgG (whole molecule) (Sigma) were used in double diffusion experiments. For Western blot analyses, alkaline phosphatase-conjugated

Abbreviations: NB, neuroblastoma; NHS, normal human serum.  
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goat anti-human IgM (μ-specific) (Sigma) was used at 1:20,000.

Cytotoxicity Assay. Complement-mediated cytotoxicity was determined by flow cytometry (FACScan; Becton Dickinson) as described (21).

Antibody Binding. Natural antibody binding to NB cells was determined by indirect immunofluorescence (21). Cells (5 x 10^5) were incubated in 100-μl NHS diluted 1:2 (vol/vol) in gelatin Dulbecco's phosphate-buffered saline [GPBS; 137 mM NaCl/2.7 mM KCl/6.5 mM sodium phosphate/1.5 mM potassium phosphate, pH 7.4/0.1% (wt/vol) gelatin] for 60 min at 0°C. The cells were washed twice in GPBS, resuspended in 100 μl of the same buffer containing the secondary antibody, and incubated for 30 min at 0°C. After three washes in GPBS, cells were resuspended in 300 μl of the same buffer and were analyzed by cytofluorimetry or fluorescence microscopy (Zeiss). To determine natural IgM binding to attached NB cells, cultivation was performed on Permanox chamber slides (Nunc). Immunofluorescent analysis was done accordingly after fixation with Strek's tissue fixative (22).

Inhibition of Natural IgM-Mediated Cytotoxicity. Cytotoxic NHS [50 μl of a 1:4 (vol/vol) dilution] was used to sensitize 2.5 x 10^5 NB cells for 45 min at 0°C in Eppendorf microfuge tubes. The cells were washed twice in GPBS and then exposed to various amounts of anti-Fcγ or -Fcα F(ab')2 fragments for 20 min at 0°C. After twice washing the cells in GPBS, complement-dependent cytotoxicity was determined with 200 μl of human sera that had been preabsorbed with 0.5 mM MgCl2 and 0.9 mM CaCl2 (GPBS^2+) for 30 min at 37°C.

Purification of IgM. NHS was chromatographed by gel filtration over Sephacryl S-300 HR (430 ml column; Pharmacia) at a flow rate of 0.1 ml/min in Dulbecco's phosphate-buffered saline (DPBS; 137 mM NaCl/2.7 mM KCl/6.5 mM sodium phosphate/1.5 mM potassium phosphate, pH 7.4) at 4°C. The column fractions (5 ml) were screened both for cytotoxic activity (see above) and the presence of IgG and IgM antigen by double diffusion technique (21). Cytotoxic fractions were pooled and dialyzed at 4°C against 8 mM phosphate, 0.1 M NaCl, pH 7.4. The pooled sample was loaded onto a 23-ml DEAE--Sephacel column (Pharmacia) and was eluted by a 0.1-1.0 M NaCl-gradient in the same buffer at a flow rate of 0.1 ml/min at 4°C.

Immunohistochemistry. Cryostat sections (5 μm) of subcutaneously grown human NB from nude rats or from a human NB patient were air-dried and fixed in chloroform-acetone (23). Immunostaining of tissue sections was done with NHS at a 1:10 dilution in DPBS containing 0.1% bovine serum albumin for 120 min. After three washes in the same buffer, the fluorescent Cy3-conjugate was added for 60 min. After postfixation with formaldehyde, counterstaining of the nuclei was performed with hematoxylin as described (23). The preparations were examined with a microscope (Zeiss) equipped for epifluorescence.

Other Methods. SDS/PAGE was performed in a minigel system (Pharmacia) (24). Immunoblotting of cell membrane extracts or IgM fractions after separation on 7% (wt/vol) or 4-20% (wt/vol) gradient SDS/PAGE and electrophoretic transfer (120 min at 50 V) onto polyvinylidene difluoride membrane (Millipore) in 3-(cyclo-hexylamino)-propyl sulfonic acid transfer buffer were performed according to established procedures (25). Cell membranes were extracted as described (14). The possible involvement of antibodies against blood group determinants was ruled out by preabsorption on test erythrocytes of various specificities (ABO, Rh, MNS, Lewis, Lutheran, Kell, Duffy, Kidd, Sex-linked; Baxter, Unterscheidheim, Germany). Lectin blots were performed with a glycan detection system as described by the manufacturer (Boeherring Mannheim). Trypsin, proteinase K, sodium periodate, and N-glycanase treatment of the NB cell membranes were performed as described (25). Natural antibody reactivity to α-Gal was ruled out by preabsorption on rabbit erythrocytes (26). GdD2, GdD3, and proteoglycan antigen 9.2.27 were ruled out as target antigens by preincubation of NB cells with monoclonal antibodies (21) and subsequent determination of natural IgM binding in a flow cytometry assay. NB tumors in nude/nude nude rats (5 weeks old) were established by s.c. injection of 2 x 10^5 LA-N-5 (n = 3) or NMB-7 (n = 2) cells (H. Juhl, unpublished work).

RESULTS

Natural Cytotoxicity of NHS Against NB Cells. Sera of 94 normal human adults were surveyed for anti-NB cytotoxicity (mean cytotoxicity, 33 ± 21%). Approximately onethird of the sera were characterized by medium-to-high cytotoxicity of 40% to 95% against cultured NB cells. (Fig. 1A). These sera are referred to as positive compared with negative sera with a cytotoxicity of <30%. Serial dilutions of positive sera revealed a significant cytotoxic effect up to a dilution factor of 1:16 (data not shown). The occurrence of cytotoxicity was independent of the individuals' age and gender. Two positive (>60% cytotoxicity) and two negative sera (<15% cytotoxicity) were selected for subsequent experiments. The positive sera were cytotoxic against all tested NB cell lines, although the extent of cytotoxicity was significantly lower for SK-N-MC cells as compared with the other NB cells (Fig. 1B). However, the positive sera lacked cytotoxic activity against malignant cell lines derived from human melanoma, lymphatic and hematopoietic malignancies (lanes 9, 10, 11, 12, 13, 14).

**FIG. 1.** Characterization of anti-NB natural cytotoxicity in NHS. (A) Natural cytotoxicity of normal human adult sera (n = 94) against LA-N-1 human NB cells. (B) Natural cytotoxicity of a positive (NHS-A, shaded bars) and a negative NHS (NHS-C, open bars) against various NB cell lines (lanes: 1, LA-N-1; 2, LA-N-5; 3, SK-N-SH; 4, NMB-7; 5, IMR-32; 6, SH-SY5Y; and 7, SK-N-MC), control tumor cell lines derived from melanoma (lanes: 8, SK-MEL-93-2; 9, SK-MEL-170), kidney (lane 10, SK-RC-29), pancreas (lane 11, Panc Tu-1), colon (lane 12, WiDr), bone marrow (lane 13, K562), lymphoblastoid (lane 14, CEM), bone marrow (lane 15, TE-85), skeletal muscle (lane 16, RD), and primary cultured normal human fibroblasts (lane 17), melanocytes (lane 18), and epidermal keratinocytes (lane 19).
poietic system, bone, colon, kidney, pancreas, and skeletal muscle (Fig. 1B). Furthermore, no cytotoxicity was observed with cultured normal human fibroblasts, melanocytes, and epidermal keratinocytes (Fig. 1B).

**Characterization of the Anti-NB Cytotoxic Factor.** The cytotoxic activity of positive sera was abolished by heat inactivation, MgEGTA treatment, and decomplementation with coba venom factor of the sera, indicating the involvement of the classical complement pathway (Table 1). Sensitization of NB cells with heat-inactivated positive serum at 0°C and subsequent incubation at 37°C in negative serum lead to a similar degree of cytotoxicity as observed with untreated positive serum (Table 1), suggesting the presence of NB-specific complement-activating antibodies in the positive sera.

This assumption was confirmed by the observation that after sensitization with positive serum at 0°C, anti-Fcμ, but not anti-Fcy specific, F(ab')2, protected NB cells from complement-mediated killing in a dose-dependent fashion (Fig. 2A). Furthermore, FACS analyses revealed binding of large amounts of IgM but only minor amounts of IgG to NB cells after incubation with positive sera (Fig. 2B). With negative sera, neither IgM nor IgG was detectable in significant amounts on the cell surface (Fig. 2B). These data are in accordance with the observation that IgM binding correlated with the degree of cytotoxicity (P < 0.001; r = 0.72; n = 70) (Fig. 2C). The specificity of IgM binding to NB cells was confirmed by the lack of IgM binding to cells derived from human melanoma, lymphatic and hematopoietic system, bone, colon, kidney, pancreas, and skeletal muscle. Furthermore, no binding was observed with cultured normal human fibroblasts, melanocytes, and epidermal keratinocytes (not shown).

Fractionation of positive serum by gel filtration revealed cytotoxic activity only in IgM-containing fractions (Fig. 3A). After further purification of the pooled IgM fractions by ion exchange chromatography, IgM was identified as the cytotoxic factor by SDS/PAGE and immunoblotting (Fig. 3B). Chromatographic fractionation of negative serum revealed no anti-NB cytotoxic activity under identical conditions. However, a five-fold concentration of the pooled IgM fractions of negative serum led to an increase in cytotoxicity from <15% to approximately 50%, suggesting that cytotoxic anti-NB IgM is present but at a significantly lower concentration (not shown).

**Cell Surface Expression of the NB Antigen.** Fig. 4 shows the binding of natural human IgM to NB cells as analyzed by indirect immunofluorescence microscopy. With detached NB cells, the fluorescent staining signal was closely associated with the plasma membrane (Fig. 4A). By changing the focus to the upper surface of the cells, a regularly clustered distribution of the staining signal became evident (Fig. 4B). A similar plasma membrane-associated signal was obtained with adherent NB cells (Fig. 4E). Using negative serum, no staining of detached or adherent NB cells was seen (Fig. 4C and F). In accordance with cytfluorometry, binding of IgM was not detected on the surface of detached or adherent melanoma cells (Fig. 4D and G).

**Characterization of the NB Antigen.** Anti-NB positive sera predominantly recognized a 260-kDa antigen in immunoblots of NB cell extracts (Fig. 5). This antigen was not recognized by negative sera. Preadsorption of the positive serum with intact NB cells reduced the staining intensity of the 260-kDa NB antigen significantly, whereas preadsorption with intact melanoma cells had no effect on the staining intensity (Fig. 5).

The recognition of the 260-kDa antigen by anti-NB IgM antibodies is susceptible to treatment of membrane extracts with proteinase K (Fig. 5, lane 4) and trypsin (not shown). There is no evidence that carbohydrate moieties are involved in the epitope structure. Both treatment with sodium periodate

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**Table 1.** Classical complement pathway dependence of anti-NB cytotoxicity in NHS

<table>
<thead>
<tr>
<th>Treatment of serum</th>
<th>% cytotoxicity*</th>
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<td>Positive serum</td>
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<tr>
<td>None</td>
<td>14</td>
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<tr>
<td>EGTA</td>
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<tr>
<td>Positive serum (heat-inactivated) +</td>
<td></td>
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<tr>
<td>negative serum (untreated effector</td>
<td>85</td>
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* Cytotoxicity was determined by cytfluorometry using propidium iodide.

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**Fig. 2.** Cell killing of human NB cells (LA-N-1) is dependent on the binding of natural IgM to the cell surface. (A) Inhibition of cell killing by various concentrations of anti-Fcμ F(ab')2 (O) in contrast to anti-Fcy F(ab')2 (C). (B) Cytfluorometric analysis of binding of natural IgM (Left) or IgG (Right) to NB cells from anti-NB-positive NHS. Negative NHS and buffer served as controls. (C) Correlation analysis of IgM binding (mean channel fluorescence intensity) and the degree of cytotoxicity.
and incubation with N-glycanase had no effect on the staining intensity (not shown). Additional lectin blot analyses with Con

**FIG. 3.** (A) Fractionation of IgM in positive NHS by gel filtration over Sephacryl S-300. Column effluent was monitored for absorbance at 280 nm (•), and fractions were assayed for both anti-NB cytotoxic activity (bars) and immunoglobulin antigens (IgM and IgG) using double diffusion technique. (B) Identification of IgM after final purification by ion exchange chromatography through 4–20% (wt/vol) gradient SDS/PAGE (silver staining) and Western blotting (alkaline phosphatase-labeled anti-human μ-chain) under both reducing (DTT +) and nonreducing (DTT −) conditions. The apparent molecular masses (in kDa) are indicated.

**FIG. 4.** Surface expression of the natural IgM target antigen on detached (A and B) and adherent (E) LA-N-5 human NB cells upon examination by indirect immunofluorescence microscopy with positive NHS. No immunoreactivity on LA-N-5 cells was seen with negative control serum (C, detached; F, adherent), and with cultured human SK-MEL-93-2 melanoma cells (D, detached; G, adherent). A Cy3 conjugate was used as secondary antibody. (×1000.)

**FIG. 5.** Western blots of LA-N-1 NB membrane extracts. Antigen detection by anti-NB positive NHS (lane 1), after preabsorption of positive NHS on human LA-N-1 NB (lane 2) or SK-MEL-93-2 melanoma (lane 3) cells. No reactivity was seen with positive NHS after proteinase K treatment of the LA-N-1 membrane extract (lane 4) and with negative NHS (lane 5). NHS dilution for primary antibody incubation was 1:10 (vol/vol).

A, peanut lectin, and *Maackia amurensis* lectin suggest that the 260-kDa antigen represents a nonglycosylated protein (not shown). In accordance with these data, several carbohydrate structures could be excluded as target antigens by cellular preabsorption or binding competition experiments with murine monoclonal antibodies. These included carbohydrate blood group determinants, the α-Gal epitope, the gangliosides GD2 and GD3, and the proteoglycan 9.2.27 (not shown). Based on the background binding and cytotoxic activity of positive sera against GM2-expressing cells such as osteosarcoma TE-85 and human fibroblasts (27, 28), GM2 can also be excluded as dominant target structure.

**Detection of the Antigen in Human NB Tumor Tissue.** Using an indirect immunofluorescence technique, extensive binding of anti-NB IgM from positive sera to cryostat sections of a human NB tumor was observed (Fig. 6A). IgM binding was not detectable with a negative serum, indicating that no endogenous patient IgM was present in the tumor (Fig. 6B). Similarly, human NB tumors established subcutaneously in nude rats from two different cell lines (n = 5) were stained only by positive serum (Fig. 6C) but not by negative serum (Fig. 6D).

**Natural Anti-NB Activity in Patient Sera.** Table 2 shows IgM

**FIG. 6.** Indirect immunofluorescence microscopy with natural anti-NB IgM-positive NHS reveals the in vivo presence of the target antigen in most cells of a fixed cryostat section of a human NB tumor (A). No immunoreactivity was evident with anti-NB negative NHS (B). Identical observations were made with a subcutaneously grown human NB derived from LA-N-5-injected nude rats (C, positive NHS; D, negative NHS). A Cy3 conjugate was used as secondary antibody. (×1000.)
binding and anti-NB cytotoxicity of five sera from newly diagnosed NB patients before chemotherapy and six sera from NB patients receiving chemotherapy. None of the sera from NB patients exhibited significant IgM binding or IgM-mediated complement killing of NB cells compared with a positive serum from a healthy adult donor. In contrast to NB patients, sera of age-matched healthy control children showed significantly higher IgM binding and anti-NB cytotoxicity with increasing age. While in group I (0–1 year) only 3% of the sera were anti-NB positive (mean cytotoxicity, 15 ± 9%; mean IgM fluorescence channel, 29 ± 27), group II (2–6 years) contained 48% anti-NB positive sera (mean cytotoxicity, 41 ± 18%; mean IgM fluorescence channel, 94 ± 66), and group III (7–14 years) 67% anti-NB positive sera (mean cytotoxicity, 48 ± 18%; mean IgM fluorescence channel, 154 ± 116) (data not shown).

DISCUSSION

Most human natural antibodies are of the IgM subclass and are mainly produced by CD5+ B cells (29, 30). However, a definite assignment of the functional role of natural antibodies is still elusive. Suggestions for their importance include the primary control of invading microorganisms (29), the clearance of immune complexes (31), and self-non-self discrimination (32). In this study, we describe the presence of cytotoxic natural IgM antibodies against human NB circulating in the sera of healthy adult individuals. The anti-NB natural IgM response is present at different levels in the tested sera, with approximately one-third exhibiting strong anti-NB reactivity. This natural IgM antibody mediates potent killing of human NB cells in vitro by activation of the classical complement pathway.

NB is characterized by a high frequency of spontaneous tumor regression. Therefore, natural IgM could theoretically provide immunological protection for the host in the case of NB development. The lack of cytotoxicity in NB patient sera supports such a hypothesis. Sera (11 of 11) obtained from patients with active disease (before and during chemotherapy) exerted only weak or background cytotoxic activity against NB cells. The result is especially intriguing in view of the finding that 8 of these patients were between 2 and 14 years old, an age group in which 60 out of 110 investigated normal healthy children (55%) exhibited strong natural IgM-mediated anti-NB activity. This percentage of positive sera is almost two-fold higher than in the normal adult population. However, more sera from patients with NB are certainly needed to correlate the occurrence of cytotoxic natural IgM with spontaneous regression of NB. Furthermore, for a reliable assessment of the phenomenon, a prospective survey of the natural anti-NB activity in NB patients is required.

All NB cell lines tested so far were positive for natural IgM binding and cytotoxicity, although at different levels (Fig. 1B). In contrast to NB cell lines, no reactivity was detected with cultured melanoma cells and melanocytes that are also of neuroectodermal origin. Various other tumor cell lines derived from bone marrow, lymphatic system, colon, pancreas, bone, skeletal muscle, and kidney, as well as cultured normal human fibroblasts and epidermal keratinocytes, also exhibited only background binding and cytotoxicity. The presence of the natural IgM epitope under in vivo conditions was confirmed with a tumor specimen from a human NB patient and with human NB tumors established in nude rats. IgM staining of NB cells in the tumor tissue was similar to the staining pattern obtained with cultured NB cells. Importantly, staining was absent in the patient tumor tissue with negative sera, indicating that the patient's serum did not contain natural anti-NB IgM consistent with the results from the other 11 NB patients (compare Table 2).

Using Western blot analysis techniques, we have identified a 260-kDa protein as target for the natural anti-NB IgM. Several analyses including periodate oxidation, N-glycanase treatment, and lectin blots suggest the absence of carbohydrates in the target protein (Fig. 5). This is in accordance with the observation that carbohydrate blood group determinants and the α-Gal epitope, both of which have been described as targets of natural antibodies (26, 29, 32), were not recognized. Some of the most abundant ganglioside structures could also be excluded as dominant target epitopes. Human melanoma cells that are known to express GD2 and GD3 (19, 21) did not bind significant amounts of natural IgM and exhibited only background cytotoxicity (Figs. 1B and 4). In addition, binding of anti-GD3 monoclonal antibodies to NB cells could not be competed for by natural anti-NB IgM of positive sera. Human TE-85 osteosarcoma cells and human fibroblasts that have been demonstrated to express significant amounts of GM2 (27, 28), exhibited also background natural IgM binding and cytotoxicity (Fig. 1B). Therefore, natural anti-NB IgM appears to be different from naturally occurring IgM antibodies with specificity for GM2 that have been shown to be present in NHS (33).

Collectively, the findings of our study provide evidence that natural humoral immunity against NB exists and is directed against a 260-kDa cell surface-associated NB protein which is expressed in vivo. Ongoing studies are aimed at characterizing the structure of the 260-kDa antigen and its possible role in clinical onset and course of NB.

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