Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis

Roberta Castriconi*†, Alessandra Dondero*, Raffaella Augugliaro‡, Claudia Cantoni*†§, Barbara Carnemolla‡, Angela Rita Sementa†, Francesca Negri†, Romana Conte‡, Maria Valeria Corrias†, Lorenzo Moretta*†§, Alessandro Moretta*§¶, and Cristina Bottino†

*Dipartimento di Medicina Sperimentale, Università degli Studi di Genova, Via L. B. Alberti 2, 16132 Genoa, Italy; †Istituto Giannina Gaslini, L.go G. Gaslini 5, 16148 Genoa, Italy; ‡Istituto Nazionale per la Ricerca sul Cancro, L.go Rosanna Benzi 10, 16132 Genoa, Italy; and §Centro di Eccellenza per le Ricerche Biomediche, Università degli Studi di Genova, V.le Benedetto XV, 16132 Genoa, Italy

Communicated by Renato Dulbecco, The Salk Institute for Biological Studies, San Diego, CA, July 12, 2004 (received for review March 26, 2004)

In this study, in an attempt to identify neuroblastoma-associated surface antigens, we generated mAbs against the ACN neuroblastoma cell line. A mAb was selected (5B14) that reacted with all neuroblastoma cell lines analyzed and allowed detection of tumor cell infiltrates in bone marrow aspirates from neuroblastoma patients. In cytofluorimetric analysis, unlike anti-disialoganglioside mAb, 5B14 mAb did not display reactivity with normal bone marrow hematopoietic cell precursors, thus representing a highly specific marker for identifying neuroblastoma cells. Molecular analysis revealed that the 5B14 mAb-reactive surface glycoprotein corresponded to the recently identified 4Ig-B7-H3 molecule. Remarkably, mAb-mediated masking of the 4Ig-B7-H3 molecule on cell transfectants or on freshly isolated neuroblastoma cells resulted in enhancement of natural killer-mediated lysis of these target cells. These data suggest that 4Ig-B7-H3 molecules expressed at the tumor cell surface can exert a protective role from natural killer-mediated lysis by interacting with a still undefined inhibitory receptor expressed on natural killer cells.

Neuroblastoma, the most common solid tumor of childhood, can arise anywhere along the sympathetic nervous system (1, 2). Frequently, the primary tumor localizes in the abdomen, with the adrenal gland being the most common site. The age of 1 year represents an important prognostic cutoff. Under 1 year of age, most tumors are localized (stages 1–2) or, if disseminated, undergo maturation into benign ganglioneuromas or regress spontaneously (the so-called stage 4S). On the contrary, most children older than 1 year of age present with a highly disseminated disease at diagnosis with metastasis involving bone marrow (BM), brain, liver, and skin (stage 4) and a very poor prognosis. Indeed, neuroblastoma is the tumor with the highest risk of death in children. In particular, because of drug resistance or relapse after conventional therapy, children at stage 4 have very poor survival rates. (The probability of 3-year survival is $<15\%$.)

One of the aims of the current research is to improve our understanding of the biological behavior of neuroblastoma and to identify markers that would be used for diagnosis, monitoring of the disease, and attempting innovative therapeutic approaches. In particular, the characterization of surface molecules expressed by neuroblastoma cells would allow a more precise identification and quantification of tumor cells, particularly in the BM, a frequent site of tumor relapses. This will improve the diagnosis and allow a better definition of the risk grade in a particular patient and the delivery of the most appropriate therapy. In this context, the disialoganglioside (GD_2) is generally used as a neuroblastoma-associated marker (3, 4). However, anti-GD_2 mAbs also react with cells other than tumor cells. A conceivable explanation is that GD_2 shed from the cell surface of neuroblastoma may bind to the surface of other cells and react with anti-GD_2-specific mAbs (5, 6). Identification of surface antigens expressed by neuroblastoma also would allow selective isolation of fresh tumor cells that could be assessed for their susceptibility to natural killer (NK)-mediated lysis. The results of these studies might provide a clue for attempting immunotherapeutic approaches based on the infusion of activated NK cells in neuroblastoma patients. Indeed, *in vitro* cultured neuroblastoma cell lines have been shown to be susceptible to NK-mediated lysis (7). It is of note that NK cells have been shown to play a central role in the cure of acute myeloid leukemias, as recently highlighted by clinical studies in which patients had received haploidentical BM transplantation (8, 9).

The aim of the present study was to identify surface markers that would allow a precise detection of neuroblastoma cells. Thanks to the generation of mAbs, we could identify the 4Ig-B7-H3 molecule (10, 11) as a valuable surface antigen for detection of neuroblastoma cells, particularly in BM aspirates. Perhaps more importantly, we also provide evidence that 41g-B7-H3 molecules play a protective role in tumor cells by inhibiting NK-mediated cell lysis. This inhibitory effect can be reversed by mAb-mediated masking of the 4Ig-B7-H3 molecule.

Methods

mAbs. 5B14 mAb (IgM) was obtained by immunizing a 5-week-old BALB/c mouse with ACN (human neuroblastoma) cell line as described (12). c218 (IgG1, anti-CD56), A6–136 (IgM, anti-HLA class I), and AZ20 (IgG1, anti-NKp30) were produced in our laboratory. The mAbs anti-GD_2 (14.G2a, IgG2A) and anti-CD45 (HI30, IgG1) were purchased from Pharmingen and Caltag (South San Francisco, CA), respectively.

Bone Marrow Aspirates and Neuroblastoma Purification. After informed consent, BM was aspirated from two iliac crests with a 1.8-gauge needle from 15 children diagnosed with neuroblastoma, admitted at the Hematology–Oncology Division of the G. Gaslini Institute, from January 2003 to January 2004. Diagnosis and staging were performed according to the International Neuroblastoma Staging System (13). The amount of BM aspirate residue after performing diagnostic and therapeutic evaluations was analyzed by flow cytometry upon red cell lysis. Neuroblastoma cells were purified from BM aspirates (from children diagnosed with neuroblastoma) by CD45 depletion by using an Enrichment of Circulating Tumor Cells kit (RosetteSep, StemCell Technologies, Vancouver).

Abbreviations: BM, bone marrow; NK, natural killer; APAAP, anti-alkaline phosphatase; PE, phycoerythrin; CHO-K, Chinese hamster ovarian carcinoma; DC, dendritic cell; GD_2, disialoganglioside.

¶To whom correspondence should be addressed. E-mail: alemoret@unige.it.

www.pnas.org/cgi/doi/10.1073/pnas.0405025101

The investigation was performed after approval by the Gaslini Institute institutional review board.

Detection of GD_2 and 5B14 Expression by Immunocytochemistry. Forty cytospins (17 mm in diameter), containing up to 5×10^5 cells per slide, were prepared from BM aspirates. Cytospins were fixed in formalin and incubated with the anti-GD_2 mAb 14.G2a (5 μg/ml, Pharmingen) or with 5B14 mouse mAb (ascitic fluid diluted 1:100) for 30 min. One slide was incubated with an isotype-matched mAb of irrelevant specificity (negative control). After washing, slides were sequentially incubated with a rabbit anti-mouse antibody (DAKO) diluted 1:20 for 30 min and an alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (APAAP) complex diluted 1:20 (DAKO) for 30 min. Alkaline-phosphatase substrate (DAKO) was subsequently added as chromogen. The slides were counterstained in hematoxylin and then cover-slipped. The results were interpreted under a light microscope.

Biochemical Characterization and Purification of 5B14-Reactive Molecule. The 5B14 mAb (IgM) was purified by using Kaptiv-M (Tecnogen, Caserta, Italy). Cells (20×10^6) were labeled with ^{125}I (NEN), lysed in 1% Nonidet P-40, and immunoprecipitated with Sepharose-CnBr (Pharmacia Biotech)-coupled 5B14 mAb. Samples were analyzed by discontinuous SDS/PAGE either undigested or digested with *N*-glycosidase F (Boehringer Mannheim). 293T cell membranes obtained as described (12) were incubated with Sepharose CnBr-coupled 5B14 mAb. Specific proteins were eluted and, upon concentration, analyzed by SDS/PAGE under nonreducing conditions. The polyacrylamide gel was stained by using Simply Blue Safestain (Invitrogen) (12).

In-Gel Enzymatic Digestion and Liquid Chromatography/ESI-MS/MS Analysis of Tryptic Peptides. In-gel digestion of the purified 5B14-reactive protein stained was carried out as described (12). Analysis of the resulting peptide mixtures was performed by LCQ-DECA MS/MS ion trap mass spectrometer coupled to an HPLC Surveyor (Thermo Finnigan, San Jose, CA) and equipped with a 1 × 150-mm column, Vydac C_{18}, 5 μm, 300 Å (Dionex) as described (12). Computer analysis of peptide MS/MS spectra was performed by using TURBOSEQUEST V. 1.2 software (University of Washington, licensed to Thermo Finnigan) and searched against the National Center for Biotechnology Information human protein database.

RT-PCR Amplification of cDNAs Encoding Human 4Ig-B7-H3 and 2Ig-B7-H3. Total RNA extracted by using peqGOLD RNA pure (PEQLAB, Erlangen, Germany) from the 293T cell line was reverse transcribed by standard technique by using oligo(dT) priming. Primers used were 5′-ATGCTGCGTCGGCGGGG (2Ig-B7-H3 UP) and 5′-GGTCAGGCTATTTCTTGTCCATC (B7-H3 DW) for 2Ig-B7-H3 and 5′-CAGCCGCCTCACAGGAAG (4Ig-B7-H3 UP) and 5′-GGTCAGGCTATTTCTTGTCCATC (B7-H3 DW) for 4Ig-B7-H3. Amplifications were performed with the hot-start technique by using AmpliTAQ (PerkinElmer). 2Ig-B7-H3 amplification (953 bp) was performed for 30 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) followed by a 7-min extension at 72°C. 4Ig-B7-H3 amplification (1,624 bp) was performed for 15 cycles (30 s at 94°C, 30 s at 58°C, and 30 s at 72°C), 15 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) followed by a 7-min extension at 72°C. PCR products were subcloned into pcDNA3.1/V5-His-TOPO expression vector (Invitrogen). DNA sequencing was performed by using BigDye Terminator cycle sequencing kit and a 377 Applied Biosystems automatic sequencer.

Soluble Molecules. 2Ig-B7-H3 (RhB7-H3/Fc chimera) was purchased from R & D Systems). The MICA soluble molecule is described in ref. 14. For the 4Ig-B7-H3Fc soluble molecule, the cDNA sequence encoding the extracellular domains (including the leader sequence) of the 4Ig-B7-H3 was amplified from codon 1 to 461 and cloned in pRB1 expression vector in frame with the cDNA sequence encoding the mutated hIgG1 as described (15). The pRB1–4Ig-B7-H3Fcmut construct was transiently transfected into a human embryonic fibroblast 293T cell line by using FuGENE 6 (Roche). Transfected cells were cultured in DMEM/10% ultra-low IgG FCS (Invitrogen), and supernatants were collected at days 4 and 8 after transfection. 4Ig-B7-H3Fc molecule was purified by affinity chromatography by using protein A Sepharose 4 Fast Flow (Amersham Biosciences). Purified protein was checked by SDS/PAGE followed by silver staining and ELISA using 5B14 mAb.

Stable Transfection. The Chinese hamster ovarian carcinoma (CHO-K) cell line was transfected with pcDNA3.1V5-His-TOPO-2Ig-B7-H3 or pcDNA3.1V5-His-TOPO-4Ig-B7-H3 construct by using GenePORTER 2 transfection reagent (GTS, San Diego) following manufacturer's instructions. After 48 h, transfected cells were selected in DMEM plus 1.5 mg/ml G418 and subcloned under limiting dilution. Selected clones were stained with 5B14 mAb followed by phycoerythrin (PE)-conjugated goat anti-mouse isotype-specific second reagent (Southern Biotechnology Associates) and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Polyclonal NK Cells. NK cells were purified by using the Human NK Cell Enrichment Mixture-RosetteSep (StemCell Technologies) and cultured on irradiated feeder cells in the presence of 100 units/ml rIL-2 (Proleukin, Chiron) and 1.5 ng/ml phytohemagglutinin (GIBCO) to obtain polyclonal activated NK cell populations.

Cytolytic Activity and Flow Cytofluorimetric Analysis. NK cells were tested for cytolytic activity against the indicated target cells in a 4-h ^{51}Cr-release assay as described (12). The concentrations of the various mAbs added for masking experiments were 10 μg/ml. The effector/target ratios are indicated in the text.

For one- or two-color cytofluorimetric analysis (FACSCalibur), cells were stained with the appropriate mAbs followed by PE- or FITC-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associates).

IFN-γ Production. IFN-γ production from polyclonal NK cells was measured in supernatants by using ELISA (IFN-γ; BioSource International, Camarillo, CA). NK cells (5×10^5 cells per ml) were incubated in 96-well U-bottom tissue culture plates either in the absence or in the presence of purified 2Ig-B7-H3 or 4Ig-B7-H3 (see above) soluble molecules or, as positive controls, in the presence of purified anti-NKp30 mAb (AZ20) or MICA soluble molecule (14) at the concentrations of 50 μg/ml or 10 μg/ml, soluble molecules and mAb, respectively.

Results

Isolation of the 5B14 mAb. In an attempt to identify cell-surface markers expressed by human neuroblastoma cells, mice were immunized with the ACN neuroblastoma cell line. After cell fusion, hybridoma supernatants were screened by indirect immunofluorescence and cytofluorimetric analysis for surface reactivity with a panel of neuroblastoma cell lines. By using this experimental approach, an mAb termed 5B14 was selected that stained not only the immunizing cells but also all of the neuroblastoma cell lines tested, including SK-N-BE (Fig. 1) and GIMEN (data not shown), that do not express GD_2, which is presently considered the most reliable marker for neuroblastoma.

We next assessed the surface distribution of the 5B14-reactive

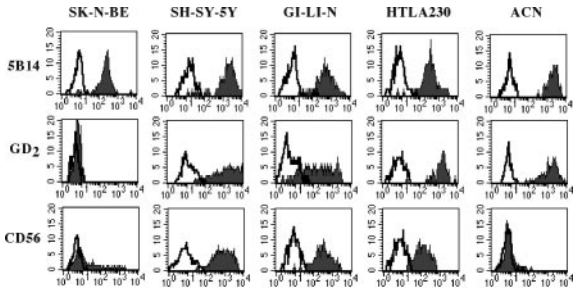

Fig. 1. Reactivity of the 5B14 mAb with neuroblastoma cell lines. Different neuroblastoma cell lines were stained with 5B14 mAb or mAbs to the indicated molecules followed by PE-conjugated goat anti-mouse isotype-specific second reagent and analyzed by flow cytometry. Open profiles indicate cells incubated with the second reagent only.

molecule(s) on normal cells and on tumor cell lines of different histotype. As shown in Table 1, 5B14 mAb did not react with either normal lymphocytes or *in vitro* cultured NK, B, and T cell lines. Low reactivity could be detected with monocytes, whereas *in vitro* induced immature or mature dendritic cells (DCs) were brightly stained. Finally, 5B14 mAb displayed a high reactivity with a large panel of tumor cell lines of different origin, including melanomas and carcinomas.

Table 1. Surface reactivity of the 5B14 mAb on different cell types

Cells	Histotype	5B14 mAb	GD2 mAb
Resting NK cells		−	−
Activated NK cells		−	−
Resting T cells		−	−
PHA blasts		−	−
Resting B cells		−	−
Granulocytes		−	−
Monocytes		+/−	−
IDC		++	−
mDC		++	−
YT	NK cell line	−	++
NK92	NK cell line	−	−
CEMB	T leukemia	−	−
Jurkat	T leukemia	−	+/−
H9	T leukemia	−	+/−
HSB2	T leukemia		
Raji	Burkitt lymphoma	−	−
LCL 721.221	EBV cell line	−	−
EA	Endothelial cells	++	−
K562	Erythroleukemia	+	−
MM6	Promyelocytic leukemia	+	−
U937	Myeloid leukemia	+	−
293T	Embryonic fibroblasts	++	−
M14	Melanoma	++	−
FO-1	Melanoma	++	++
Me 1074	Melanoma	++	++
H460	Lung carcinoma	++	−
SMMC	Hepatoma	++	−
HELA	Cervical carcinoma	++	−
IGROV-1	Ovarian carcinoma	++	−
SKNEP	Kidney carcinoma	+	−
A172	Glioblastoma	++	−
HT29	Colon carcinoma	++	−
CX2	Colon carcinoma	++	−
SKBr3	Breast carcinoma	+	−
MDAM B453	Breast carcinoma	+/−	−

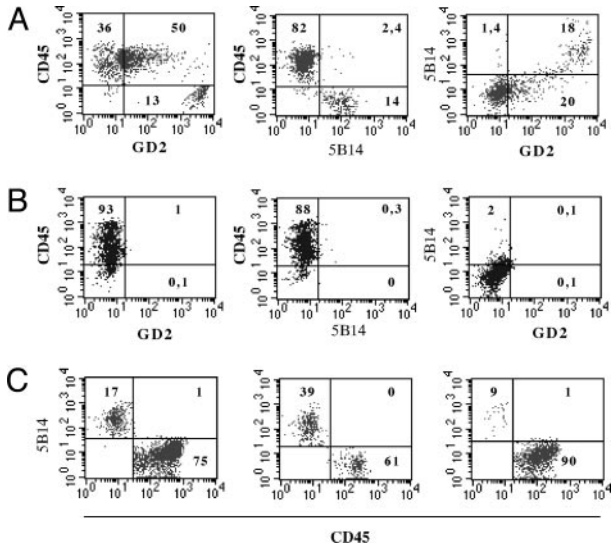

Fig. 2. Comparative cytofluorimetric analysis of 5B14 and GD_2 reactivity on freshly derived neuroblastoma cells. Two representative BM aspirates derived from children affected either by neuroblastoma at stage 4 (*A*) or at nonmetastatic stage 1 (*B*) were analyzed by double fluorescence and cytofluorimetric analysis with 5B14 mAb or mAbs to the indicated molecules followed by PE- or FITC-conjugated goat anti-mouse isotype-specific second reagent. Three additional representative stage 4 BM aspirates characterized by a different number of infiltrating neuroblastoma cells are shown in *C*. Statistical analysis (in terms of % of positive cells) is indicated.

Cytofluorimetric Analysis of Fresh Neuroblastoma Bone Marrow Infiltrates. Bone marrow aspirates were derived from 16 children affected by neuroblastoma at stage 4 or (as a control) from 15 patients at the nonmetastatic stage 1. Samples were analyzed by double fluorescence and cytofluorimetric analysis using either 5B14 or anti-GD_2 mAbs (Fig. 2). In stage 4 samples, the anti-GD_2 mAb brightly stained the infiltrating $CD45^-$ neuroblastoma cells (Fig. 2*A*). It is of note, however, that anti-GD_2 mAb also reacted with a high proportion of $CD45^+$ normal cells. In agreement with the hypothesis that anti-GD_2 mAb may bind soluble GD_2 molecules shed by the neuroblastoma cell surface (5, 6), anti-GD_2 surface reactivity on normal cells was not detectable in samples that did not contain neuroblastoma cells, i.e., in BM derived from nonmetastatic stage 1 patients (Fig. 2*B*). In stage 4 BM aspirates, the infiltrating neuroblastoma cells were specifically recognized by the 5B14 mAb (Fig. 2*A*). Remarkably, in no instances were 5B14-reactive molecules detected in normal $CD45^+$ cells (Fig. 2*A*), as it occurs for GD_2. Moreover, 5B14 surface reactivity was clearly detected regardless of the number of neuroblastoma cells infiltrating the different stage 4 BM aspirates (Fig. 2*C*).

Immunocytochemical Analysis of Fresh Neuroblastoma Bone Marrow Infiltrates. Bone marrow samples from patients diagnosed as having stage 4 neuroblastoma were analyzed by means of the immunocytochemical assay standardized by the European International Society of Paediatric Oncology immunocytology/genetics group, which is based on the detection of GD_2 (see *Methods*). Cytospins prepared with the same method from the same BM samples were tested in parallel with the 5B14 mAb. Two independent observers assessed the light microscopic features of the results. Quantitative evaluation was carried out on GD_2-positive cases according to the defined minimal criteria of positivity agreed on by the European International Society of Paediatric Oncology immunocytology/genetics group. The results were then compared with those yielded by the 5B14 tests on the same BM samples. The staining method applied with the two

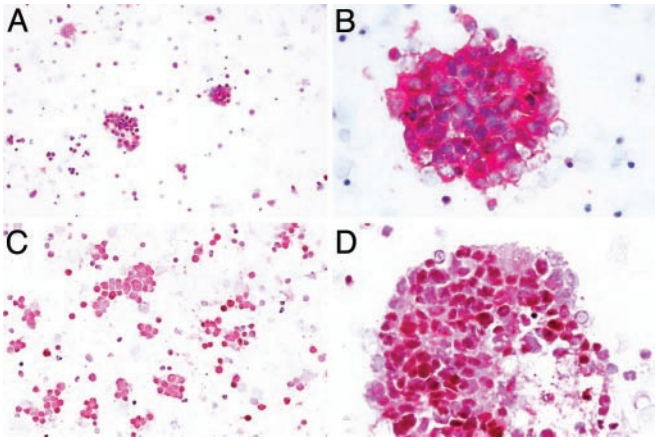

Fig. 3. Immunohistochemical detection of GD_2 and 5B14 positive cells in BM aspirates from cases of stage 4 neuroblastoma. (*A*) Cytospin preparation of BM aspirate (mAb GD_2, APAAP, enlargement ×20) immunostaining of single cells and clumps of neuroblastoma (NB) cells. In the background, fragments showing some immunostaining. (*B*) Detail at higher magnification (mAb GD_2, APAAP, ×63); the brightness of the immunostaining for mAb GD_2 can mask the morphological features of the cells, thus interfering with the recognition and the quantitative evaluation of the number of NB cells. (*C*) Cytospin of BM aspirate from the same sample as in *A* (mAb 5B14, APAAP, ×20). (*D*) Detail at higher magnification (mAb 5B14, APAAP, ×63) of a clump of cells from the same BM aspirate as in *B*. The subtler and weaker (than GD_2) quality of immunostaining is visible against a somewhat cleaner background. The morphological features of the nuclei are readily discernible.

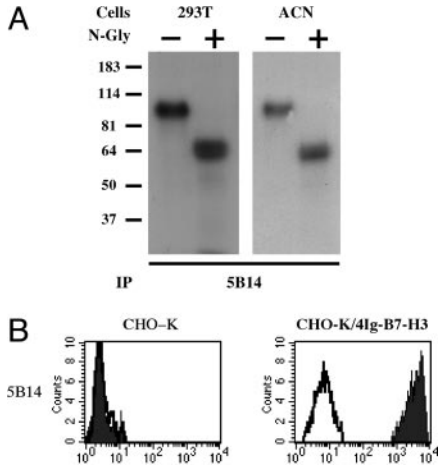

Fig. 4. Biochemical characterization of the 5B14-reactive molecule and cytofluorimetric analysis of cell transfectants. (*A*) 293T and ACN cell lines were surface-labeled with ^{125}I and immunoprecipitated with the 5B14 mAb. Samples, either untreated (−) or treated (+) with *N*-glycosidase F, were analyzed in an 8% SDS/PAGE under reducing conditions. Molecular mass markers (kDa) are indicated. (*B*) CHO-K cells either untransfected or transfected with the 4Ig-B7-H3 cDNA were stained with 5B14 mAb followed by PE-conjugated goat anti-mouse isotype-specific second reagent and analyzed by flow cytometry. Open profiles indicate cells incubated with the second reagent only.

mAbs showed similar numbers of positive cells. Although in most samples, GD_2 was highly and consistently expressed by neuroblastoma cells and not normal leukocytes, background staining was present especially in preparations containing a high number of neuroblastoma cells, such as those from most stage 4 patients at the onset of the disease (Fig. 3). This effect may interfere with positive cell discrimination and counting. Cytospins from the same cases tested with 5B14 mAb showed almost identical detection sensitivity, whereas nonspecific or background staining was significantly less or absent (Fig. 3). In all cases the quality of staining with 5B14 mAb, although somewhat weaker or less brilliant, turned out to be decidedly more clear-cut and did not usually mask the nuclear profile or morphological details of the cells. The staining quality contributes to easier identification of the morphological hallmarks of the cells, which also plays a role in the overall evaluation of the BM samples.

Biochemical Characterization of the Surface Molecule Recognized by 5B14 mAb. The representative 293T (embryonic fibroblasts) and ACN (neuroblastoma) human cell lines were surface-labeled with ^{125}I, and cell lysates were immunoprecipitated with 5B14 mAb. In both cases, 5B14 mAb immunoprecipitated a surface molecule of ≈100 kDa both under reducing (Fig. 4*A*) and nonreducing (data not shown) conditions. The protein backbone remaining after treatment with *N*-glycosidase F displayed a molecular mass of ≈60 kDa, thus predicting the existence of numerous N-linked glycosylations. 5B14-reactive molecules were purified from 293T cell membranes by affinity chromatography. In-gel tryptic digested molecules were analyzed by liquid chromatography-MS/MS. Three different peptides allowed the identification of the 5B14-reactive molecule. It was found to correspond to a transmembrane protein of 534 aa characterized by four Ig-like domains in the order V/C/V/C (10). This molecule has been recently termed 4Ig-B7-H3 (11). In line with the biochemical analysis above, the prediction for *N*-glycosylation sites showed height NxS/T consensus sequences. 4Ig-B7-H3 is encoded on human chromosome 15 (15q23-q24) and appears to be the result of a gene duplication of the exons encoding the IgV-IgC domains (10). It is of note that the human B7-H3 was originally described as a molecule characterized by two Ig-like domains in the order V/C (16). However, whereas 2Ig-B7-H3 represents the unique form found in mice, in humans it is likely to represent a short form generated by alternative splicing (10).

To unequivocally confirm the identity of the 5B14-reactive molecule, CHO-K cells transfected with 4Ig-B7-H3 cDNA were analyzed for surface reactivity with the 5B14 mAb. In all instances, no reactivity could be detected in untransfected CHO-K cells. Conversely, 5B14 mAb strongly reacted with 4Ig-B7-H3 cell transfectants (Fig. 4*B*). According to the high percentage of identity between the distal and membrane proximal V/C domains (11), 5B14 mAb also recognized 2Ig-B7-H3 cell transfectants (data not shown).

4Ig-B7H3 Molecules Inhibit Human NK Cell Cytotoxicity. It is possible that 4Ig-B7-H3 expressed at the neuroblastoma cell surface may exert an inhibitory or enhancing effect on the NK-mediated recognition and/or lysis of the tumor cells. To evaluate this possibility, we analyzed the cytolytic activity of *in vitro* cultured human NK cells against the CHO-K cell line either untransfected or transfected with 4Ig-B7-H3 molecules. As shown in Fig. 5*A*, NK cells efficiently lysed untransfected CHO-K cells. In this case, addition of 5B14 mAb had neither inhibitory nor enhancing effect on the cytolytic activity. On the contrary, lysis of 4Ig-B7-H3-transfected CHO-K cells was strongly reduced. Moreover, lysis could be restored in the presence of 5B14 mAb. The resulting lysis was similar to that of untransfected CHO-K cells. These results on cell transfectants strongly suggested that 4Ig-B7-H3 molecules also could exert a protective role in the case of neuroblastoma cells. To verify this possibility, additional experiments were performed by using as target cells freshly purified neuroblastoma cells derived from five different BM aspirates of stage 4 patients. These cells were $CD45^-$ and displayed a bright fluorescence for GD_2, 4Ig-B7-H3 (Fig. 5*B*), and CD56 (data not shown); i.e., they were characterized by a surface phenotype typical of neuroblastoma (17). Neuroblastoma cells were virtu-

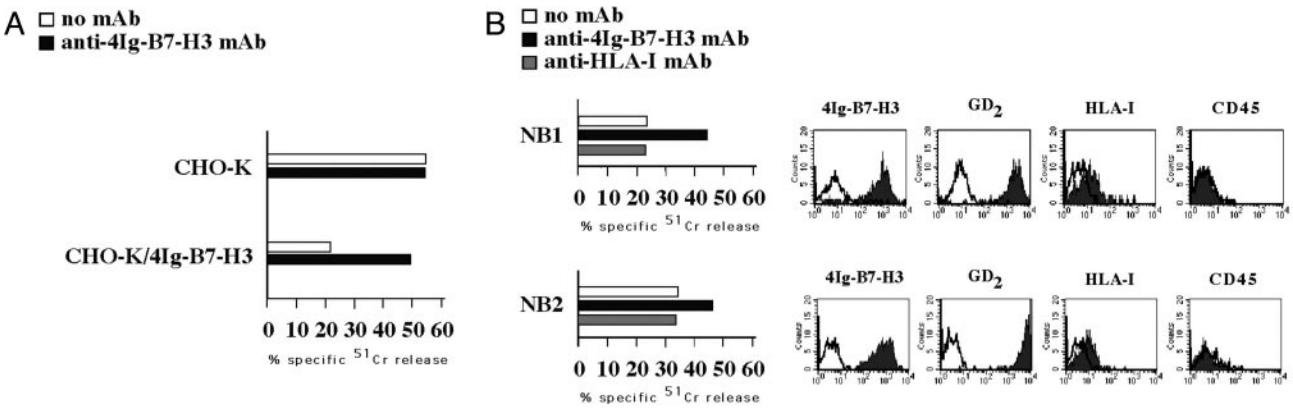

Fig. 5. 4Ig-B7-H3 molecules protect target cells from NK-mediated cytotoxicity. A polyclonal NK cell population was analyzed for cytolytic activity against CHO-K or 4Ig-B7H3-CHO-K transfectants (*A*) or against two representative freshly purified neuroblastoma cell populations (NB1 and NB2) (*B Left*) either in the absence of mAb or in the presence of mAbs to 4Ig-B7-H3 (5B14) or HLA class I (A6–136). The effector/target ratio used was 2:1 (*A*) or 20:1 (*B*). The results are representative of three independent experiments; SD of the mean of the triplicates was <5%. Note that neuroblastoma cells were highly homogeneous as indicated by the fact that 4Ig-B7-H3 and GD_2 molecules were expressed by the entire cell population (flow cytometry of NB1 and NB2 in *B Right*). Open profiles indicate cells incubated with the second reagent only.

ally negative for the expression of HLA class I molecules (Fig. 5*B*) known to protect target cells from the NK-mediated attack by interacting with specific inhibitory NK receptors (18). Accordingly, mAb-mediated masking of HLA class I molecules had no enhancing effect on the NK-mediated lysis of neuroblastoma cells (Fig. 5*B*). Conversely, addition of 5B14 mAb resulted in a substantial enhancement of cytolysis (Fig. 5*B*). These data clearly indicate that 4Ig-B7-H3 molecules are capable of down-regulating NK cell-mediated cytotoxicity, thus protecting neuroblastoma from the NK-mediated attack. As a corollary, NK cells should necessarily express a still undefined 4Ig-B7-H3-specific receptor exerting inhibitory functions. This hypothesis is further substantiated by the fact that the engagement of this putative receptor by either 2Ig-B7-H3 or 4Ig-B7-H3 soluble molecules did not induce IFN-γ production in polyclonal NK cells, whereas the engagement of classical triggering NK receptors such as NKp30 or NKG2D (14) resulted in abundant cytokine release (Fig. 6). Thus, although an initial report suggested a costimulatory function of the putative B7-H3 receptor on T cells (16), our present findings imply that in human NK cells, it may exert inhibitory rather than activating function.

Discussion

The present study provides two important insights into neuroblastoma cell biology and the relationship of this tumor with NK-mediated immune responses. First, we identified 4Ig-B7-H3 (10, 11) as a surface marker that is specific for neuroblastoma, at least in BM aspirates. Second, we show that this molecule, belonging to the B7 family, inhibits the NK-mediated lysis of neuroblastoma by interacting with a still-undefined receptor expressed by NK cells. The present data may have a noticeable impact on improving the diagnosis of neuroblastoma and, possibly, in future attempts of new therapeutic approaches.

The 5B14 mAb, obtained by mice immunization with a neuroblastoma cell line, was found to react not only with all of the available neuroblastoma cell lines, but also with all tested freshly isolated neuroblastoma cells. 5B14 mAb allows detection, with high specificity, of tumor cells in BM aspirates from patients with metastatic, stage 4 neuroblastoma. Indeed, by double fluorescence and fluorescence-activated cell sorter analysis, 5B14 mAb selectively stained CD45-negative tumor cells, whereas anti-GD_2 mAb, currently used for neuroblastoma cell identification, displayed some reactivity also with $CD45^+$ normal hematopoietic cells. Moreover, purified CD45-negative neuroblastoma cells were homogeneously stained by 5B14 mAb. These data, together with the finding that 5B14, unlike anti-GD_2 mAb (5, 6), stained all of the neuroblastoma cell lines analyzed, indicated that 5B14 mAb represents a valuable reagent to precisely identify neuroblastoma cells and to discriminate between tumor and normal cells in BM aspirates.

As revealed by molecular analysis, 5B14-reactive molecules could be identified with 4Ig-B7-H3, a member of the B7 family that has been described very recently (10, 11). The surface expression of 4Ig-B7-H3 protected tumor cells from NK-mediated killing. Thus, 4Ig-B7-H3 cell transfectants were more resistant to lysis than the corresponding untransfected target cells. In addition, 5B14 mAb-mediated masking of 4Ig-B7-H3 molecules resulted in efficient killing of cell transfectants by NK cells. These data also suggest that NK cells express receptor(s) that, upon engagement with 4Ig-B7-H3 molecules on neuroblastoma cells, deliver inhibitory signals resulting in down-regulation of NK cell cytotoxicity. This finding has been confirmed also in freshly isolated neuroblastoma cells because their lysis mediated by NK cells could be up-regulated in the presence of 5B14 mAb. Thus, it is possible to speculate that the surface expression of

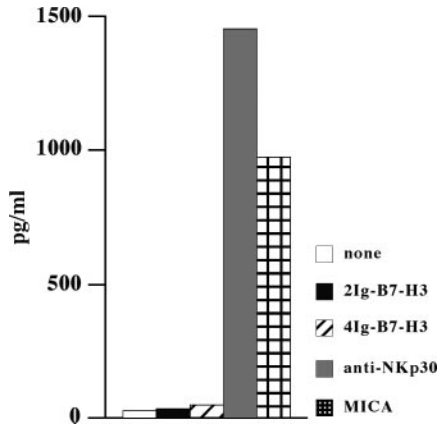

Fig. 6. B7-H3 soluble molecules do not induce IFN-γ production by NK cells. A representative polyclonal NK cell population from a healthy donor was stimulated or not with plate-bound soluble molecules (2Ig-B7-H3, 4Ig-B7-H3, and MICA) or the anti-NKp30 mAb, and IFN-γ production was assessed by ELISA. Data are representative of four independent experiments.

4Ig-B7-H3 may provide an additional mechanism allowing neuroblastoma cells to escape the control of immune response. Notably, most fresh neuroblastoma cells do not express surface HLA-class I molecules (this report), thus escaping detection by cytolytic T lymphocytes. However, on losing HLA-class I, they become potentially susceptible to NK-mediated lysis (19). In this context, the expression of 4Ig-B7-H3, capable of inhibiting NK cell function, could represent a further mechanism of evasion by which neuroblastoma also would escape the NK-mediated control. Because 4Ig-B7-H3 is not restricted to neuroblastoma but is also expressed by other tumors including melanomas and carcinomas, it is conceivable that the 4Ig-B7-H3-mediated functional inhibition of NK cells may represent a more general mechanism of tumor escape from NK cells.

4Ig-B7-H3 is a member of the growing B7 family of cell-surface ligands (20–22). These include B7–1 (CD80), B7–2 (CD86), B7-H2 (ICOS-L), B7-H1 (PD-L1), B7-DC (PD-L2), B7-H4 (B7S1, B7x), and BT3. They are specifically recognized by receptors displaying either activating (CD28 and ICOS) or inhibitory function (CTLA-4, PD-1, and BTLA) (20–22) and are mainly expressed by T lymphocytes. Thus, the putative inhibitory receptor specific for 4Ig-B7-H3 would represent the first receptor for a B7 family member to be expressed by human NK cells. It is of note that an initial report suggested that the putative 2Ig-B7-H3-specific receptor exerted a costimulatory function during human T cell activation (16). These data, however, are not supported by our present results and also were challenged by recent studies in human (11) and mouse T cells (21, 22). Indeed, engagement of the putative receptor for 4Ig-B7-H3 expressed on human T cells did not result in costimulatory function, whereas the mouse 2Ig-B7-H3-specific putative receptor has been shown to function as a negative regulator for TH1 responses. Thus, it seems conceivable that the 4Ig-B7-H3-specific receptor may exert an inhibitory rather than a stimulatory function in both T and NK cell-mediated responses. Conversely, at present it is not possible to rule out that, similar to other members of this family (23), two distinct receptors for B7-H3 may exist that are capable of transducing either positive or negative signals, respectively.

It is of note that 4Ig-B7-H3 molecules are highly expressed by monocyte-derived immature or mature DC (this report and ref. 11). A number of recent studies have highlighted the role of the cross-talk occurring between NK and DC in the early phases of innate immune responses and in shaping the subsequent T cell responses toward the TH1 phenotype (24). Thus, one might speculate that NK-mediated recognition of 4Ig-B7-H3 molecules at the DC surface may play a regulatory role during NK/DC interactions.

Our present data suggest that anti-4Ig-B7-H3 mAb may represent a most reliable diagnostic tool, allowing specific detection of tumor cells in BM, a primary site of neuroblastoma tumor relapses. Moreover, they could offer a clue for future development of NK cell-based (8, 9), therapeutic approaches in neuroblastoma and, possibly, in other tumors expressing 4Ig-B7-H3 molecules.

We thank Drs. E. Lanino, A. Garaventa, and B. De Bernardi (Istituto G. Gaslini, Genova) for providing neuroblastoma BM aspirates; Dr. E. Bogenman (Children's Hospital, Los Angeles) for the generous gift of the HTLA-230 cell line; and Drs. L. Zardi, C. Gambini, and G. Melioli (Istituto G. Gaslini, Genoa) for fruitful discussions. This work was supported by grants awarded by Associazione Italiana per la Ricerca sul Cancro, Istituto Superiore di Sanità, Ministero della Sanità, Ministero dell'Università e della Ricerca Scientifica e Tecnologica, European Union QLRT-2000-01495 Project "Therapeutic antibodies" Consiglio Nazionale delle Ricerche, and Progetto Finalizzato Biotecnologie, Fondazione Italiana per la Lotta al Neuroblastoma, and Fondazione Compagnia di San Paolo, Torino, Italy. F.N. is the recipient of a fellowship awarded by Fondazione Italiana per la Lotta al Neuroblastoma.

1. Brodeur, G.-M. (2003) *Nat. Rev. Cancer* **3,** 203–216.
2. Schwab, M., Westermann, F., Hero, B. & Berthold, F. (2003) *Lancet Oncol.* **4,** 472–480.
3. Schulz, G., Cheresh, D.-A., Varki, N.-M., Yu, A., Staffileno, L.-K. & Reisfeld, R.-A. (1984) *Cancer Res.* **44,** 5914–5920.
4. Hakomori, S. (1984) *Annu. Rev. Immunol.* **2,** 103–126.
5. Ladish, S., Wu, Z.-L., Feig, S., Schwartz, E., Floutsis, G., Wiley, F., Lenarsky, C. & Seeger, R. (1987) *Int. J. Cancer* **39,** 73–76.
6. Valentino, L., Moss, T., Olson, E., Wang, H.-J., Elashoff, R. & Ladisch, S. (1990) *Blood* **75,** 1564–1567.
7. Sivori, S., Parolini, S., Marcenaro, E., Castriconi, R., Pende, D., Millo, R. & Moretta, A. (2000) *J. Neuroimmunol.* **107,** 220–225.
8. Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.-D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., *et al.* (2002) *Science* **295,** 2097–2100.
9. Velardi, A., Ruggeri, L., Moretta, A. & Moretta, L. (2002) *Trends Immunol.* **23,** 438–444.
10. Sun, M., Richards, S., Prasad, D.-V., Mai, X.-M., Rudensky, A. & Dong, C. (2002) *J. Immunol.* **168,** 6294–6297.
11. Steinberger, P., Majidic, O., Derdak, S.-V., Pfistershammer, K., Kirchberger, S., Klauser, C., Zlabinger, G., Pickl, W.-F., Stockl, J. & Knapp, W. (2004) *J. Immunol.* **172,** 2352–2359.
12. Bottino, C., Castriconi, R., Pende, D., Rivera, P., Nanni, M., Carnemolla, B., Cantoni, C., Grassi, J., Marcenaro, S., Reymond, N., *et al.* (2003) *J. Exp. Med.* **198,** 557–567.
13. Brodeur, G.-M., Pritchard, J., Berthold, F., Carlsen, N.-L., Castel, V., Castelberry, R.-P, De Bernardi, B., Evans, A.-E., Favrot, M., Hedborg, F., *et al.* (1993) *J. Clin. Oncol.* **11,** 1466–1477.
14. Andre, P., Castriconi, R., Espeli, M., Anfossi. N., Juarez, T., Hue. S., Conway. H., Romagne, F., Dondero, A., Nanni, M., *et al.* (2004). *Eur. J. Immunol.* **34,** 961–971.
15. Falco, M., Marcenaro, E., Romeo, E., Bellora, F., Marras, D., Vely, F., Ferracci, G., Moretta, L., Moretta, A. & Bottino C. (2004). *Eur. J. Immunol.* **34,** 1663–1672.
16. Chapoval, A.-I., Ni, J., Lau, J.-S., Wilcox, R.-A., Flies, D.-B., Liu, D., Dong, H., Sica, GL., Zhu, G., Tamada, K. & Chen, L. (2001) *Nat. Immunol.* **2,** 269–274.
17. Komada, Y., Zhang, X.-L., Zhou, Y.-W., Inaba, H., Deguchi, T., Azuma, E. & Sakurai, M. (1998) *Cancer* **82,** 591–599.
18. Moretta, A., Bottino, C., Mingari, M.-C., Biassoni, R. & Moretta, L. (2002) *Nat. Immunol.* **3,** 6–8.
19. Garrido, F., Ruiz-Cabello, F., Cabrera, T., Pérez-Villar, J.-J., Lòpez-Botet, M., Duggan-Keen, M. & Stern, P.-L. (1997) *Immunol. Today* **18,** 89–95.
20. Carreno, B.-M. & Collins, M. (2002) *Annu. Rev. Immunol.* **20,** 29–53.
21. Chen, L. (2004) *Nat. Rev. Immunol.* **4,** 336–347.
22. Moretta, A. & Bottino, C. (2004) *Eur. J. Immunol.* **34,** 2084–2088.
23. Suh, W.-K., Gajewska, B.-U., Okada, H., Gronski, M.-A., Bertram, E.-M., Dawicki, W., Duncan, G.-S., Bukczynski, J., Plyte, S., Elia, A., *et al.* (2003) *Nat. Immunol.* **4,** 899–906.
24. Moretta, A. (2002) *Nat. Rev. Immunol.* **2,** 957–965.