A Novel Metastatic Animal Model Reflecting the Clinical Appearance of Human Neuroblastoma: Growth Arrest of Orthotopic Tumors by Natural, Cytotoxic Human Immunoglobulin M Antibodies

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ABSTRACT

Neuroblastoma (NB), the most common extracranial solid tumor in childhood is associated with poor prognosis in patients with advanced tumor stages. Natural human cytotoxic anti-NB IgM antibodies present in the serum of healthy humans are discussed as a potential novel immunotherapeutic regimen against human NB because these antibodies have been shown to affect growth arrest of solid s.c. xenografts of human NB in nude rats. Subcutaneously induced tumors, however, exhibit a different growth pattern compared with the typical growth pattern of NB tumors in humans. Therefore, we developed in this study a novel metastatic tumor model in nude rats that reflects the clinical appearance of human NB and used this model to study the therapeutic efficacy of human anti-NB IgM. Intra-aortal injection of human NB cells in nude rats resulted in the development of large invasive adrenal gland tumors and micrometastases in the liver and bones. Apparently, adrenal glands provide most favorable growth conditions for human NB cells, as documented by the preferential and rapid growth of NB cells in this location. We studied three different treatment protocols of natural human anti-NB IgM. Anti-NB IgM completely inhibited tumor formation and metastases when injected simultaneously with human LAN-1 NB cells (P < 0.05). When antibody treatment was started 6 days after tumor cell injection (i.e., micrometastatic stage), tumor growth was inhibited by 90% (P < 0.05). An anti-NB IgM therapy directed against established tumors (14 days after tumor cell injection) shrank adrenal gland tumors by 90% (P < 0.05). Analysis of the tumors revealed both complement activation and an induction of apoptosis as two independent mechanisms of antitumor function. This study strongly suggests human anti-NB IgM antibodies as new agents for the therapy of neuroblastoma.

INTRODUCTION

NB is the most common extracranial solid tumor in childhood. During the past two decades the 5-year survival rate has increased from 48% because of better staging methods and improved chemotheraphy. However, the prognosis is still poor, with an overall 5-year survival rate of 67% and depends on tumor stage, localization, and the presence of risk factors such as age of patient >1 year and N-myc amplification (1). The development of new treatment strategies becomes obligatory, especially for children with advanced tumor stages. Within the last 15 years cytotoxic antibodies have been shown to enable tumor cell lysis in vitro and in vivo by activating antibody-dependent cellular cytotoxicity and a complement-dependent cytotoxicity (2–5). Meanwhile, antibodies directed against the GD2 antigen are in Phase III trials and show promising results (6).

Recently, natural human cytotoxic anti-NB IgM antibodies have been discovered in the serum of healthy humans. High levels of these antibodies were reported in ~30% of blood donors (7). Interestingly, these antibodies are barely found in children suffering from neuroblastoma, suggesting a protective role in neuroblastoma development (8).

Natural human anti-NB IgM antibodies recognize a M, 260,000 surface protein on human neuroblastoma cells (7, 9). A pilot study has demonstrated the efficacy of these antibodies in eradicating s.c. xenografts of human NB in nude rats (10). Despite the use of an extremely small amount of active IgM antibody and the large size of the antibody, which theoretically hinders its tissue distribution, a dramatic tumor regression was achieved. This is unlikely to solely rely on an antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity activation, and indeed it was found that these antibodies also induce apoptosis, which significantly contributes to their cytotoxicity (9, 10).

The principal mechanism of human anti-NB IgM antibody function in vivo has been determined in s.c. xenograft models. However, comparative studies of s.c. and orthotopically injected tumor cells demonstrate that the resulting tumors differ regarding their invasive and metastatic behavior (11–13). A more realistic view of the clinical potential of anti-NB antibodies could be obtained if a tumor model that better reflects the growth pattern of advanced NB in children (i.e., large adrenal gland tumors and multiple small metastatic lesions preferentially in the liver and bones) were available.

In this study we have characterized a new human NB animal model that reflects the common growth pattern of NB in children. Using this model we demonstrate the ability of natural human cytotoxic anti-NB IgM antibodies to eradicate human NB cells.

MATERIALS AND METHODS

Cell Culture. Human LAN-1 NB cells were obtained from R. C. Seeger (University of California, Los Angeles, CA) and cultivated in RPMI 1640 (Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS.

Purification of Human Anti-NB IgM. Serum of blood donors was obtained from the Transfusion Medical Center of the University Hospital Kiel and screened for the presence of cytotoxic anti-NB antibodies using an in vitro cytotoxicity assay as previously described (7). Two donors were identified who showed anti-NB cytotoxicity of >90% cell killing (donors 005 and 062). The IgM fraction of the serum of these donors was purified, and cytotoxicity was confirmed. In this study we exclusively used the serum from donor 005, which showed a cytotoxicity level of 94% of LAN-1 cell lysis.

Purification of the IgM fraction was performed by a combination of size exclusion (Sephacryl S-300 HR; Amersham Pharmacia Biotech, Freiburg, Germany) and anion exchange chromatography (Macro-Prep High Q; Bio-Rad, Munich, Germany) as previously described (7). Using this procedure, IgM was purified to homogeneity as confirmed by SDS-PAGE and Western blotting as previously described (8). Purified fractions were concentrated to 1 mg/ml PBS by ultrafiltration with stirred ultrafiltration cells (Amicon, Beverly, MA). Aliquots were stored at 4°C after sterile filtration through a 0.2-μm cellulose acetate filter. Antibody binding and the cytotoxicity against NB cells were confirmed by FACS analysis using dichlorotriacinylamino-fluorescein-
conjugated goat antihuman IgM (Dianova, Hamburg, Germany) and propidium iodide, respectively (7).

**Animal Experiments.** Four-week-old male nude rats (nu/nu) were obtained from Harlan Winkelmann (Borchen, Germany). To induce metastatic NB, rats were anesthetized, and $1 \times 10^7$ LAN-1 NB cells in a 50-$\mu$L volume were injected in the tail vein or after laparotomy, in the capsule of the left adrenal gland or in the aorta abdominals next to the diaphragm. The lethality of either method was 0%. Bleeding occurred in 5% of rats receiving an intra-aortal cell injection and was controlled intraoperatively by local compression. Antibodies were injected i.p. as described in detail in “Results.” At the end of the experiments rats were killed and inspected, and adrenal glands, kidney, femur, liver, and lung were removed, snap-frozen in liquid nitrogen, and stored at $-80^\circ$C until further studied. Adrenal gland tumors were measured in three dimensions, and the volume was calculated by the formula $V = \frac{d_1 \times d_2 \times d_3}{2}$.

**Tissue Immunostaining.** Cryostat sections (5 $\mu$m) of the respective organs were prepared. As a staining method, we used the immunoperoxidase technique as previously described (14). NB cells were identified by murine monoclonal antibodies directed against the NB84 antigen (DAKO, Glostrup, Denmark) and the GD2 antigen (BW 704; Behringwerke, Marburg/Lahn, Germany). Additional staining included monoclonal antibodies directed against p53 (DAKO), Fas-receptor (Immunotech, Marseilles, France), and Fas-ligand (Alexis Corp., Grünberg, Germany). In parallel to an immunostaining we performed H&E staining for morphological evaluation.

**Determination of the Proliferation Rate.** Tumors were immunostained using the MIB-1 antibody purchased from Dianova (Hamburg, Germany) and the immunoperoxidase technique. In a blinded manner two independent investigators counted stained tumor cells of 20 microscopic fields to determine the proliferation rate ($=$ percentage of proliferating cells within a tumor).

**Determination of the Apoptotic Rate.** Tumors were stained using the Apo Tag TUNEL staining kit obtained from Oncor (Gaithersburg, MD). Two independent investigators counted stained tumor cells of 20 microscopic fields to determine the apoptotic rate ($=$ percentage of apoptotic cells within a tumor).

**Detection of IgM Antibodies within NB Tissue.** Tumor cryostat sections of IgM antibody-treated and untreated (buffer control) animals were stained by immunofluorescence technique using a fluorescent Cy3-labeled rabbit antihuman IgM antibody (Dianova, Hamburg, Germany) as previously described (9).

**Detection of Complement Accumulation in Adrenal Gland Tumors.** Cryostat slices from adrenal glands of IgM-treated and control animals were immunostained using polyclonal goat anti-C3 antibodies (Quidel, San Diego, CA) and alkaline phosphatase-labeled rabbit antigoat IgG antibody (Sigma Chemical, Deisenhofen, Germany) as previously described (9).

**Data Analysis.** For statistical analysis we used the unpaired $t$ test.

**RESULTS**

**Establishment of a Human NB Tumor Model in Nude Rats**

**Comparison of Intra-adrenal (Orthotopic), Tail Vein, and Intra-aortal Injection**

LAN-1 NB cells ($1 \times 10^7$) were injected in the capsule of the left adrenal gland ($n = 3$), the tail vein ($n = 4$), and subdiaphragmal in the aorta ($n = 6$). The animals were examined weekly under anesthesia, and tumor growth was compared.

After 3 weeks a laparotomy was performed and showed large adrenal gland tumors of $5,300 \pm 1,100$ mm$^3$ (mean $\pm$ SD) in orthotopically injected rats and $1,570 \pm 450$ mm$^3$ in the intra-aortal group. Tail vein injection did not cause visible tumors at this time interval. Five weeks after orthotopic injection, extremely large encapsulated adrenal gland tumors $25,132 \pm 1,570$ mm$^3$ were found in all of the animals of the orthotopically treated animals. One hundred percent of the rats that were injected in the tail vein or in the aorta developed infiltrative adrenal gland tumors. Although tail vein injection resulted in tumors of $42 \pm 12$ mm$^3$, intra-aortal injection caused tumors of $5300 \pm 1300$ mm$^3$. Regardless of the type of injection, adrenal tumors showed the typical appearance of human NB with a gray to yellow color, hemorrhage, and foci of calcification.

Macroscopically, metastases were not apparent in either group, but femur fracture occurred frequently in rats that had bone metastases identified histologically. We prepared tissue sections of various organs (femur, liver, kidney, lung, spleen) and searched for micrometastatic tumors using immunohistochemistry (GD2 and NB84 antibodies). None of the rats receiving orthotopic injection of LAN-1 cells presented micrometastatic tumor growth or even single disseminated NB cells. After tail vein injection we found in one rat a single cluster of micrometastatic NB cells in the femur whereas all of the other organs remained tumor cell free. Most frequently metastases appeared in rats receiving intra-aortal cell injection. Multiple micrometastases (cell clusters of >10 cells) were found in 50% (3 of 6) of the livers and in 60% (4 of 6) of the femurs. The femur of all four mice showed both bone marrow involvement and bone-infiltrating tumor growth. Neither micrometastases nor single cells were found in the lung, kidney, and spleen of this group. Figure 1 shows a NB tumor in situ, the histopathological picture of the adrenal gland and femur metastases (H&E staining), and immunostaining of a liver micrometastasis by anti-GD2 antibodies.

From these data we decided to use the intra-aortal injection modus for further studies because it best reflects the typical growth pattern of human NB within the shortest period of time.

**Time Course of Tumor Growth**

In the next set of experiments we analyzed the time course of metastatic NB growth. The data are summarized in Fig. 2. After intra-aortal injection of $1 \times 10^7$ LAN-1 cells, animals were killed after 1, 3, 7, 14, and 21 days. The adrenal glands, liver, and femur were asservated, and cryostat sections were prepared and immunostained using NB84 and GD2 antibodies. After 24 h, 3 days, and 7 days we found increasing numbers of tumor cells in the adrenal glands and to a lesser extent in the liver and bone. Fourteen days after tumor cell injection small adrenal tumors became visible, which grew to a size of 6–8 mm in diameter after 21 days. In the femur and the liver multiple micrometastases appeared after 21 days.

**Comparison of Adrenal Gland and Liver/Femur Tumors**

To evaluate the effect of the local environment on tumor growth we compared the proliferation and apoptotic rates in adrenal gland tumors and liver or bone metastases by MIB-1 immunostaining and TUNEL staining, respectively. Adrenal gland ($n = 8$), liver ($n = 4$), and bone tumors ($n = 4$) showed a proliferation rate of 80 $\pm$ 5.1% (mean $\pm$ SD), 78 $\pm$ 6.8%, and 79 $\pm$ 12.6%, respectively ($P > 0.05$). In contrast, the apoptotic rates between adrenal gland tumors and liver/bone tumors differed significantly ($P < 0.05$). Although $5 \pm 1.7$% of the tumor cells in adrenal glands underwent apoptosis, 50 and 60% of NB cells were apoptotic in the liver and femur, respectively (SDs, $\pm 4.8$ and $\pm 10.1$, respectively). Further immunostaining including p53, Fas-receptor, and Fas-ligand oncoproteins did not reveal any differences in the expression of these molecules between adrenal gland and liver/bone tumors (Table 1).

**Cytotoxic Effects of Human Anti-NB IgM Antibodies in Vivo**

**Purification and Characterization of Human Anti-NB IgM Antibodies**

We screened blood of 74 blood donors for the presence of cytotoxic anti-NB IgM antibodies and identified two donors with high cytotoxic antibody levels (>90% cell killing of LAN-1 cells in vitro). For the
experiments in this study, we purified IgM exclusively from the serum of one donor with a high cytotoxic antibody level (94% cell killing).

Analysis of the isolated IgM fraction by SDS-PAGE and Western blot revealed highly purified antibodies (Fig. 3A), which efficiently bind to NB cells (Fig. 3B) and lyse >90% of LAN-1 cells (Fig. 3C). We also confirmed binding to the Mr 260,000 antigen using Western blot (data not shown).

**Treatment of NB in Vivo**

We analyzed the effect of human anti-NB IgM antibodies at different stages of metastatic tumor growth. On the basis of our time course of tumor development we performed three experiments to analyze the antibody effect in relation to the tumor stage. We investigated two different adjuvant situations: before the onset of tumor cell proliferation in the adrenal gland (treatment directly after tumor cell injection) and after the appearance of micrometastatic cells in the adrenal gland stroma (treatment 6 days after cell injection). In a third experiment we studied antitumor effects in established metastatic adrenal gland NB tumors.

**Experiment 1 (Early Adjuvant Therapy).** In this experiment we started the therapy of rats (n = 5) directly after tumor cell injection. Every other day, rats received 1 mg of purified IgM antibodies eight times i.p. The treatment was discontinued 16 days after cell injection, and on the 22nd day animals were killed and inspected, and organs were removed for analysis.

None of the antibody-treated rats showed enlarged adrenal glands. Not even a single tumor cell in the gland was detectable, as determined by immunostaining, which suggests a complete eradication of the injected tumor cells (Fig. 4A).

**Experiment 2 (Late Adjuvant Therapy).** Animals (n = 4) received the same therapy regimen as it was applied in experiment 1, but treatment started 6 days after tumor cell injection, and animals were killed 1 day after the final antibody injection (on the 22nd day). All four rats developed small adrenal gland tumors with a mean total adrenal gland volume of 95 ± 21.04 mm³ (mean ± SD). This macroscopic finding was confirmed by histological and immunohistochemical evaluation. Single tumor cells were present in one liver and two femur samples as detected by anti-NB84 and anti-GD2 immunostaining (Fig. 4A).

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**Table 1** Staining results of adrenal gland tumors and neuroblastoma micrometastases in the femur and liver with respect to the apoptotic (TUNEL staining) and proliferation rate (MIB-1 immunostaining).

<table>
<thead>
<tr>
<th>Tumor location</th>
<th>Apoptotic ratea</th>
<th>Proliferation rate</th>
<th>p53b</th>
<th>Fas receptorb</th>
<th>Fas ligandb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>5% (SD ± 1.7)</td>
<td>80% (SD ± 5.1)</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Liver</td>
<td>50% (SD ± 4.8)</td>
<td>80% (SD ± 6.8)</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Femur</td>
<td>60% (SD ± 10.1)</td>
<td>80% (SD ± 12.6)</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
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a The difference of the apoptotic rate between adrenal gland and liver/femur tumors was statistically significant (P < 0.05).
b Immunostaining for the apoptosis-associated proteins p53, Fas-receptor, and Fas-ligand was performed. ++, fair staining (20–50% of cells); –, no staining.
In control experiments ($n = 4$) rats were treated with buffer, and tumors reached a mean volume of $870 \pm 460 \text{ mm}^3$. Micrometastatic cell clusters were found in one liver and two femurs. The difference in tumor sizes of experiment 1 and experiment 2 compared with the control group was statistically significant ($P < 0.05$). The number of positive liver and femur samples was too small to allow statistical evaluation.

**Experiment 3 (Treatment of Advanced Tumors).** Two weeks after intra-aortal tumor cell injection, rats ($n = 4$) with visible adrenal gland tumors received 1 mg of anti-NB IgM antibodies for 5 consecutive days. A control group ($n = 4$) was similarly treated with buffer solution. The rats were killed 24 days after tumor cell injection (i.e., 5 days after the final antibody injection).

All of the rats of the control group developed large NBs in both adrenal glands, with an average tumor volume of $2821 \pm 1200 \text{ mm}^3$. Significantly smaller tumors ($P < 0.05$) were found in animals treated with IgM antibodies, showing an average tumor volume of $318 \pm 109 \text{ mm}^3$ (Fig. 4B). In both the treatment group and the control group, liver micrometastases were present in one rat of each group. One animal of the treated versus three rats of the control group showed micrometastases in the femur.

**Evaluation of the Cytotoxic Antibody Effects**

**Detection of IgM Antibodies in Adrenal Gland Tumors**

To determine whether the injected antibodies reached their target, we performed cryostat sections of adrenal gland tumors of the treatment group and the control group of experiment 2. Slides were stained using a fluorescent Cy3-labeled rabbit antihuman IgM antibody. Our analysis show accumulation of IgM antibodies within the tumor tissue and tumor cell membrane staining in animals treated with purified anti-NB IgM antibodies. IgM staining was not found in tumor-free regions of the adrenal gland and was also undetected in adrenal gland tumors of untreated animals (Fig. 5).

**Complement-Activation in Antibody-treated Adrenal Gland Tumors**

One potential mechanism of action of cytotoxic anti-NB IgM antibodies is the activation of the classical pathway of complement, which results in an accumulation of the complement component C3. Using anti-C3 immunostaining, we compared IgM- and buffer-treated tumors and observed a significant deposition of C3 in all of the IgM-treated tumors, whereas tumors of the control group were virtually negative for C3. Figure 6, A and B, shows the results of the immunostaining, which is representative for the comprehensive analysis of all of the tumors.
bodies inhibit in vivo DISCUSSION

IgM antibody-treated animals was 29-fold higher apoptotic rate than the adrenal gland tumors. This same proliferation rate (80%), but liver and bone tumors presented a 6-fold lower apoptotic rate than the liver and bone tumors. This strongly suggests that the stromal microenvironment promotes progression of NB cells in the adrenal glands because comparable numbers of NB cells were able to reach the stroma of adrenal glands, liver, and bones. The low apoptotic rate in adrenal glands suggests most favorable growth conditions for NB cells, whereas the liver and femur may present a more hostile environment, which still enables survival but slows down tumor growth. Although the factors that are responsible for these differences are not known, our model may help to elucidate them in future studies.

We used the intra-aortal injection modus to study the therapeutic effect of natural anti-NB IgM antibodies because it reflects the growth pattern of metastatic human NB and offers the longest observation period. Together with a low mortality and the absence of lethality, this approach justifies to us the slightly increased stress for the animals.

Because NB cells preferentially formed large metastatic tumors in the adrenal gland, our main interest was the eradication of NB cells from this location because antibodies have to attack tumor cells in a place that offers them most favorable growth conditions.

We demonstrated a remarkable therapeutic effect against established NB, which in the adrenal glands shrank by almost 90%. Additionally, we tested the ability of anti-NB IgM antibodies to block the development of metastatic disease in early stages of progression. We applied antibodies before homing of tumor cells to the adrenal glands (directly after tumor cell injection) and when tumor cells had reached the stroma of target organs (6 days after cell injection). Metastatic growth was completely inhibited in the first group, but even after micrometastases had formed within adrenal gland stroma, the growth significantly slowed down, and tumors reached ~10% of the size of the untreated control group.

The high potential of anti-NB IgM antibodies is fully appreciated when one considers the injected amount of active antibody. The antibody doses of 1 mg/day is comparable with other studies using anti-GD2 antibodies (2, 3). However, although these studies applied monoclonal antibodies, we used the total IgM fraction, which is assumed to contain only 0.1–1% (±1–10 μg) of specific IgM, in other words ~100- to 1000-fold less antibody than other antibody studies. This strongly suggests that anti-NB IgM antibodies activate not only common complement pathways but have further specific cytotoxic properties. We confirmed previous studies (10) and showed that in addition to complement activation, the apoptotic rate was increased 6-fold in antibody-treated rats. Thus far, neither the antigen nor the apoptotic pathway of anti-NB IgM antibodies have been characterized, and further research will focus on their elucidation.

Previously we have demonstrated that human anti-NB IgM antibodies inhibit in vivo tumor growth of s.c. xenografts (10). In this study we characterized a new animal model that was designed to reflect the clinical situation of NB and enables analysis of the therapeutic potential of these antibodies under conditions that allow human NB cells to develop their typical aggressive and metastatic growth.

Nude rats were chosen because rat and human complement share comparable properties when activated by human antibodies (15). Furthermore, the size of nude rats simplifies surgical manipulation compared with nude mice. A disadvantage of nude rats is that T cells, which may cause tumor rejection, begin to recover after 12 weeks (16). This limits the time frame of therapeutic studies and makes it important to develop a model in which metastatic NB appears within a short period of time.

In our study, orthotopic injection led to large adrenal gland tumors but did not cause metastatic growth. In contrast, adrenal gland tumors and metastases appeared when LAN-1 cells were injected either in the aorta or, though significantly smaller, in the tail vein. The appearance of large adrenal gland tumors is remarkable because approximately <1% of tumor cells may have directly reached the adrenal gland after intra-aortal injection and even less after tail vein injection. Various mechanisms may contribute to the development of adrenal gland tumors, such as optimal homing conditions of the endothelium and a microenvironment that supports tumor growth. The finding of an additional micrometastatic spread exclusively in the liver and bones (femur) supports such mechanisms that appear to be similar in rats and humans, where children with NB typically present huge adrenal gland tumors and multiple small metastases preferentially in the bones and liver (17, 18).

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From a theoretical point of view these antibodies have two major advantages compared with murine antibodies derived against tumor-associated antigens. Because of their human origin they are not immunogenic, and more importantly, the chance of major cytotoxic side effects is remote because they naturally occur in humans.

Natural human cytotoxic anti-NB IgM antibodies have a high potential to improve current therapy of human NB. To us it becomes obligatory to bring them into clinic. However, for clinical purposes antibodies must be prepared in a large scale. Therefore, we currently prepare monoclonal antibodies by recombination techniques, which can be applied in clinical studies and help in understanding their function as highly effective anti-NB agents.

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