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Molecular Characterization of the Anti-Idiotype Immune Response of a Relapse-Free Neuroblastoma Patient following Antibody Therapy: A Possible Vaccine against Tumors of Neuroectodermal Origin?1

Martina M. Uttenreuther-Fischer,2* Jörg A. Krüger,2* and Peter Fischer3*†

Neuroblastoma treatment with chimeric disialoganglioside GD2 Ab ch14.18 showed objective antitumor responses. Production of anti-idiotype Abs (Ab2) against ch14.18 (Ab1) in some cases was positively correlated with a more favorable prognosis. According to Jerne’s network theory, a subset of anti-idiotype Abs (Ab2β) carries an “internal image” of the Ag and induces Abs (Ab3) against the original Ag. The molecular origin of an anti-idiotype Ab response in tumor patients was not investigated previously. To clone anti-idiotype Abs, B cells of a ch14.18-treated neuroblastoma patient with Ab2 serum reactivity were used to construct Ab phage display libraries. After repeated biopannings on ch14.18 and its murine relative, anti-GD2 mAb 14G2a, we selected 40 highly specific clones. Sequence analysis revealed at least 10 of 40 clones with different Ig genes. Identities to putative germline genes ranged between 94.90 and 100% for Vh and between 93.90 and 99.60% for Vk. An overall high rate of replacement mutations suggested a strong Ag-driven maturation of the anti-idiotype Abs. Two clones that were analyzed further, GK2 and GK8, inhibited binding of ch14.18 to GD2 just as the patient’s serum did. GK8 alone inhibited >80% of the patient’s anti-idiotype serum Abs in binding to ch14.18. Rabbits vaccinated with GK8 or GK2 (weaker) produced Ab3 against the original target Ag GD2. GK8 may be useful as a tumor vaccine for CD3-positive tumors. The Journal of Immunology, 2006, 176: 7775–7786.

The outcome of the advanced or relapsed stage IV neuroblastoma is still dismal regardless of continuing therapeutic efforts. Drawbacks of an intensification of chemotherapy include secondary malignancies or therapy-related deaths due to the increased toxicity of such a regimen (1–3). Passive immunotherapy with murine or human/mouse chimeric Abs directed against GD2, a disialoganglioside overexpressed on neuroblastoma cells, in phase II/III trials demonstrated complete remissions and prolonged event-free survival in some neuroblastoma patients (4–15). Initially, the clinical efficacy of native anti-GD2 Ab therapies was mainly attributed to their capability of causing tumor cell killing by Ab-dependent, cell-mediated cytotoxicity and complement-dependent cytotoxicity (16). However, although the infused Abs were cleared from circulation after six half-lives, which is ~17 days in children for mAb ch14.18, clinical remissions were of longer duration, implying that additional antitumor defense mechanisms must have been triggered (17, 18). The potential role of a “vaccination-like” effect in long-term neuroblastoma survivors suggested by Cheung et al. and other research teams (4, 10, 19–21) indicated that patients with an immune response benefited from passive immunotherapy. Similar results were confirmed for the adult patient population (22–24).

Human anti-mouse Abs (HAMAs)4 seemed to preclude repetitive therapeutic use of murine Abs by a diminished tumor targeting, accelerated clearance, and reduction of direct anti-tumor effects (9, 20, 25–27). Some of these problems were addressed by decreasing the size and the xenogeneic protein parts of Abs (28, 29). However, even with chimerized or humanized Abs, immunogenicity was still observed, although it neither limited treatment nor made patients prone to increased toxicity (14, 30–32).

In an analysis of a larger patient population of neuroblastoma survivors, Cheung et al. (6) proposed the hypothesis that low, transient levels of HAMAs, which are mainly directed against the constant regions of murine Abs, were positively correlated with patient survival. Immunogenicity of diagnostic or therapeutic mAbs was not disadvantageous for the patient but appeared beneficial by triggering an activation of the idiotype network, as proposed by Jerne in 1974 (33). Accordingly, Ag-specific idiotype (therapeutic) Ab (Ab1) induces production of the anti-idiotype antibody (Ab2) by unique antigenic determinants of its Ag combining site.

Anti-idiotype Ab2s include three groups of Abs: 1) Ab2α, which binds to the variable region of Ab1 but does not fit into the paratopes of Ab1 or inhibit Ag-binding; 2) Ab2β, which forms a mirror or internal image of the three-dimensional structure and the interactions of the Ag recognized by Ab1 (34); and 3) Ab2γ, which inhibits Ag binding but does not mirror the Ag. Ab2β itself can induce an immune response termed Ab3, the so-called anti-anti-idiotype Ab, which also binds the original tumor-associated Ag. Therefore, Ab2β can be used as a surrogate Ag, e.g., as a vaccine.

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2 M.M.U.-F. and J.A.K. contributed equally to this work and should be considered first authors.

3 Address correspondence and reprint requests to Dr. Peter Fischer, University of Applied Sciences, Fachbereich V Life Sciences and Technology, Biotechnology, Seestrasse 64, 13347 Berlin, Germany. E-mail address: pfischer@tfh-berlin.de

4 Abbreviations used in this paper: HAMA, human anti-mouse Abs; Ab1, idiotype (therapeutic) Ab; Ab2, anti-idiotype Ab; Ab3, anti-anti-idiotype Ab; BMT, bone marrow transplant; IVIG, pooled human i.v. Ig; R/S, ratio of replacement to silent mutations; scFv, single-chain variable fragment.

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Numerous clinical trials using Ab2β as an antitumor vaccine were performed or are currently underway, investigating Ab3 development and clinical remissions (35–41). Interestingly, in contrast to some of the above studies (22, 23, 41–45), Cheung et al. (46) found that high HAMA levels prevented Ab3 formation in neuroblastoma patients treated with anti-GD2 mAbs. Compiling results from previous studies they found the following: 1) patients with more intensive chemotherapy pretreatment before immunotherapy had lower and usually only transient HAMA/Ab2-levels; 2) Ab3 production, starting ~6–14 mo after mAb-therapy and persisting over years, was positively correlated with improved outcome, i.e., prolonged event-free survival, and was more pronounced in patients with lower and only transient HAMA/Ab2-levels (46); and 3) high HAMA concentrations were counterproductive for survival, as they limited efficacy of further treatment cycles. In conclusion, Cheung et al. speculated that heavy chemotherapy eradicated lymphoid structures, as reflected by low HAMA/Ab2-responses, and eliminated suppressor T cell pathways and B cells. Ab2 might then bias the recovering immune repertoire toward the GD2 network (20, 46–48).

Moreover, Cheung et al. (46, 49) assumed that high Ab2 concentrations were not inductive for Ab3. In the murine model only IgM led to Ab3 production, and IgG was rather suppressive. In a healthy immune system, dominant B cell clones (Ab2) may prevent an anti-idiotypic response (Ab3) against themselves by an early Ig class switch from immunogenic IgM to suppressive IgG class before anti-idiotypic B cells (anti-Ab2) are activated.

Currently two Ab2β-vaccines are used for the treatment of GD2-positive malignancies, i.e., 1A7, a murine Ab2β against 14G2a, and 4B5, created by murine/human heteromyeloma technology and directed against 14G2a as well (36, 50–53). Proof of principle was provided by all studies, i.e., clinical responses and Ab3 serum levels in melanoma and neuroblastoma patients treated with 1A7, and positive Ab3 levels in animals treated with 4B5 (50, 52, 53). However, although Foon et al. (36) did find Ab3 IgM in some of their melanoma patients, in general there was a positive correlation between IgM and IgG levels of Ab3, and isolated high Ab3 IgM levels were accompanied by disease progression (50). Because the hypotheses and experimental findings presented above are partially contradictory, Cheung et al. (20) suggested early on that more insight into the activation of the idiotypic network should be obtained by cloning such anti-idiotypic Abs directly at the B cell level; instead, most of the clinical studies that speculated on an activation of the idiotypic network were driven by Ab2 and Ab3 levels detected in the sera of patients treated with either Ab1 or Ab2.

In the present study we report a set of 40 human anti-idiotypic Abs against ch14.18, cloned for the first time directly from B cells of a patient after ch14.18-treatment by Ab phage display technology. Two Ab2 clones, which were investigated further, elicited an Ab3 response in rabbits, and at least one may be suitable as a tumor vaccine for GD2-positive malignancies. To the best of our knowledge, this work provides the first analysis of the molecular origin of the Ig repertoire toward the GD2 network (20, 46–48).

Materials and Methods

Patient selection

Sera for anti-idiotypic screening were obtained from nine high risk neuroblastoma patients treated with ch14.18 according to the NB97 protocol of the Society for Pediatric Oncology and Hematology (German abbreviation GPOH) (www.kinderkrebsinfo.de/el662/el7722/c5406/index frm.html) at the Charité Children’s Hospital Berlin, Germany (28, 54). Serum sampling was performed after informed consent was obtained from all patients or their guardians, respectively, according to the Helsinki Declaration. Initial screening of patients’ sera was done on 14G2a, a murine anti-GD2 Ab sharing the same variable region with ch14.18, but it was never given to our patients therapeutically (55). Patients’ sera were also tested on OKT3, a murine anti-CD3 mAb and a member of the IgG2a subclass like 14G2a (S. Gillies, unpublished observation) (56). Because the amino acid sequence of the framework region of OKT3 and 14G2a are identical, non-specific binding of HAMAs and the anti-framework Abs that only react with epitopes of 14G2a outside the CDRs could be largely excluded.

Screening assay for Ab2

Microtiter plates (Costar; Corning) were coated with 14G2a (250 ng/well), a murine anti-GD2 Ab (BioTechnocells) in PBS at 4°C overnight. After washing with PBS containing 0.2% casein, plates were blocked with PBS containing 1% casein for 1 h at 37°C. Serial dilutions of patient sera in PBS were added and incubated for 1 h at 37°C. After washing with PBS, bound anti-idiotypic and potential anti-mouse Ig Abs were detected by a peroxidase-labeled, goat anti-human Fc fragment (Jackson ImmunoResearch Laboratories). ELISAs were performed twice, and samples were tested in duplicate or triplicate.

GD2 binding inhibition by Ab2 in patients’ sera

Microtiter plates were coated with GD2 (Pierce) dissolved in methanol at a concentration of 150 ng/well for 2 h until the methanol had evaporated. Plates were blocked and washed as described above. Patient samples were added at serial dilutions together with biotinylated ch14.18 (250 ng/well) and incubated at 37°C for 1 h. ch14.18, a chimeric anti-GD2-mAb (BioInvent) was exclusively supplied for i.v. mAb therapy. Leftover i.v. supplies were used for in vitro studies. For biotinylation, a commercial kit (Sigma-Aldrich) was used following the manufacturer’s instructions. After washing with PBS, bound ch14.18 was detected with peroxidase-labeled streptavidin (Jackson ImmunoResearch Laboratories).

Determination of Ig isotypes and subclasses of anti-idiotypic antibodies

Microtiter plates coated with GD2 were overnight at 4°C with 700 ng of 14G2a in PBS per well. Plates were blocked and washed as described above. Patient serum (patient 1) containing anti-idiotypic Abs was added at a dilution of 1:250, for 1 h at 37°C. After washing with PBS, biotinylated Abs against the different Ig isotypes and IgG subclasses (BD Pharmingen) as well as against α and λ chains (DakoCytomation) were applied according to the manufacturer’s recommendations. Pooled human i.v. IgG (IVIG) (Sandoglobulin; Chiron-Behring) was used as a control.

GD2 binding inhibition by purified Fab

Microtiter plates were coated with GD2, blocked, and washed as described above. Purified Fabs were added at dilutions between 0 and 1.5 μg together with biotinylated ch14.18 (250 ng/well) and incubated at 37°C for 1 h. After washing with PBS, bound ch14.18 was detected with peroxidase-labeled streptavidin. Purification of Fab is described in detail below in this section.

Competition assay of purified Fab and patient serum (Ab2β) on 14G2a

Microtiter plates were coated with 14G2a as described above. Purified Fabs were added at concentrations between 0 and 6 μg/well together with the serum of patient 1 at a dilution of 1/20,000 to adjust for IgG concentration and incubated at 37°C for 1 h. After washing with PBS, bound anti-idiotypic and potential anti-mouse Ig Abs of the patient were detected using a peroxidase-labeled goat anti-human Fc Ab (Jackson ImmunoResearch Laboratories).

Binding of Ab3 from rabbit serum to GD2

Microtiter plates were coated with GD2. The IgG content of the different rabbit sera was determined by a sandwich ELISA using a coating of anti-rabbit IgG/IgM/IgA (BioTez) and a secondary anti-rabbit IgG HRP Ab (DakoCytomation). A serial dilution of a rabbit-anti-goat Ab served as a standard (Jackson ImmunoResearch Laboratories). For calculations, the program Revelation (Bio-Rad) was used. A rabbit serum volume containing ~35 μg of rabbit IgG was added to each well. Sera from the three rabbits immunized with Fab phase were preincubated for 2 h at 37°C on a microtiter plate precoated with pooled human Abs (1 μg/well). Thereafter, supernatants were transferred to GD2-coated microtiter plates, incubated for 1 h at 37°C, and washed with PBS. Bound rabbit Abs were detected using peroxidase-labeled goat anti-rabbit IgG (DakoCytomation). Ch14.18 served as a positive control and was developed by a peroxidase-labeled, anti-human Fab (Pierce, via KMF).
RNA preparation and library construction

Four Ab phage display libraries, IgG1 κ, IgG1 λ, IgG2 κ, and IgG2 λ, were constructed using the PBLs of patient 1, collected 30 days after his fifth ch14.18 treatment according to published protocols (57, 58) with minor modifications (59, 60).

Biopanning procedure on 14G2a and ch14.18

To increase the number of different Fab phages at the beginning of the biopanning, all four libraries were produced separately and mixed (IgG1 κ together with IgG2 κ and IgG1 λ together with IgG2 λ) just before the first biopanning cycle. The 14G2a/ch14.18-binding Fab phages were selected by the panning of 1013 recombinant phages in Maxisorp immunotubes (Nunc) coated with 300 μl of 14G2a or ch14.18 at a concentration of 30 μg/ml and blocked with 1% casein. Unbound phage was removed, and the tubes were washed vigorously up to 10 times with 0.2% casein in PBS. Then, the bound phages were eluted with 300 μl of 0.1 M HCl/glycine (pH 2.2) and neutralized with 60 μl of 2 M Tris-HCl (pH 9). Eluted phages were used to infect 3 ml of a fresh Escherichia coli XLI-Blue culture grown with tetracycline. After a 20-min incubation at 37°C on a shaker at 240 rpm, the number of eluted phages was determined by plating dilutions.

Large-scale production of Fabs in E. coli

To remove the gene III fragment for Fab expression in E. coli, cells of a selected clone in 20 ml of super broth medium containing 50 μg/ml carbenicillin, 20 μg/ml kanamycin, grown for 1 h as described above, and then further diluted in 100 μl of super broth with 50 μg/ml carbenicillin. After a 1-h incubation, 1011 VCSM13 helper phages were added. Following another 2-h incubation, kanamycin was added, and the culture was grown overnight. Panning on 14G2a/ch14.18 was repeated three more times (59, 60).

Purification of selected Fabs from E. coli supernatant prepared as described above or by Fischer et al. (61). The V regions of selected Fabs were determined from phage or plasmid DNA purified with either Midiprep (Qiagen) or Wizard columns (Promega). Sequencing was done in an automatic sequencer (LI-COR) using the cycle sequencing kit (Amersham Biosciences) and infrared fluorophore (IRD41)-labeled primers. The dye-labeled sequencing primers were PEB and SeqGb for the H chains and OmpA and SeqLb or SeqKb for the L chains (57, 63, 64). V-BASE (version 16.12; 1997) (65) was searched via the internet (vbase.mrc-cpe.cam.ac.uk) using the program DNA-Plot (version 2.0.1; developed by W. Müller and H.-H. Althaus, University of Cologne, Cologne, Germany) for the determination of germline segments and mutations.

Vaccination of rabbits with Ab2-Fab and Fab phage

Animal experiments were conducted by Biomed Research. Two separate experimental settings were assessed. In group 1 two animals were immunized intradermally with 200 μg of purified GK2 Fab (rabbit 1) and GK8 Fab (rabbit 2) in PBS using CFA. The animals were fortnightly boosted subcutaneously with 100 μg of Fab in IFA. In group 2, three animals were immunized with GK2 Fab phage, GK8 Fab phage, and anti-BSA Fab phage, respectively. Phages (5 × 1011) were used for each injection. No additional adjuvant was applied. The animals were also boosted fortnightly.

Serum samples were obtained before the first immunization and every two weeks thereafter, until day 42. The final bleed took place after 3 months.

Results

Selection of patient 1 and characterization of his anti-variable region Abs

A total of 66 serum samples from nine patients, drawn at various time points during ch14.18-treatment, were available for testing. Samples had been obtained as early as day 1 after the first ch14.18-treatment up until day 275 following the sixth Ab therapy. Sera of three of nine patients (patients 1, 6, and 8) contained anti-14G2a variable region Abs of various levels (initial assay not shown). None of the serum samples tested were reactive with OKT3, an Ab with the same framework region as 14G2a. Binding to 14G2a, but not to OKT3, was a condition (but not proof) for containing Ab2 internal image Abs. Serum IgG concentrations were measured before the assessment of 14G2a and OKT3 reactivity to exclude possible artifacts that may arise simply from very high IgG concentrations.

Serum of patient 1 revealed significant anti-variable region Ab reactivity at all IgG concentrations as depicted in (Fig. 1a). However, even at high protein concentrations it did not bind to OKT3 (Fig. 1b). Antivariable region Abs in patient 1 showed a clear increase after repetitive ch14.18-treatments, indicating a booster effect (data not shown).

Abs interfering with the paratope, such as internal image Abs to ch14.18 (Ab2β) or Ab2γ, are required to inhibit binding of
exclusively of IgG isotype, IgG1 and IgG2 subclass for the H Abs of patient 1 were further characterized in ELISAs. Abs were to construct specific Ab phage display libraries, the anti-idiotypic Combinatorial phage display libraries at the binding site of ch14.18. Serum of a normal donor did not inhibit binding at all (Fig. 2). These results were a further indication (but still not proof) that the Serum of a neuroblastoma patient showed only some GD2 binding inhibition at low serum dilutions of up to 1/4. whereas the serum of another neuroblastoma patient, who had been treated with ch14.18 at another institution and therefore was not included in this study, showed very little binding inhibition.

ch14.18 to target Ag GD2. The serum of patient 1 completely inhibited GD2 binding of ch14.18 at dilutions of up to 1/256, whereas the serum of another neuroblastoma patient showed only some GD2 binding inhibition at low serum dilutions of up to 1/4. Serum of a normal donor did not inhibit binding at all (Fig. 2). These results were a further indication (but still not proof) that the serum of patient 1 may contain internal image Abs (Ab2) aiming at the binding site of ch14.18.

**Combinatorial phage display libraries**

To construct specific Ab phage display libraries, the anti-idiotypic Abs of patient 1 were further characterized in ELISAs. Abs were exclusively of IgG isotype, IgG1 and IgG2 subclass for the H chains, and both the κ and λ L chains had been used (data not shown).

Library sizes, i.e., the maximum number of different clones, of the four libraries constructed were 9.1 × 10^6 for IgG1 κ, 3.4 × 10^6 for IgG1 λ, 6.2 × 10^6 for IgG2 κ, and 6.7 × 10^6 for IgG2 λ. The presence of complete H and L chain inserts was analyzed in 10 clones randomly picked per library; 100% of the IgG1 κ, 70% of the IgG1 λ, 70% of the IgG2 κ, and 60% of the IgG2 λ library contained both inserts as estimated by DNA-restriction from single colonies and also from total phagemid DNA.

**Biopanning of phage**

Four biopannings were performed. Corresponding κ and λ libraries were mixed. The IgG1 κ library, together with the IgG2 κ library, was selected on either ch14.18 (panning I) or 14G2a (panning II); the IgG1 λ and IgG2 λ libraries were panned on either ch14.18 (panning III) or 14G2a (panning IV).

Panning I and II showed a continuous strong increase of specifically binding phage during all four cycles of biopanning. The phage output/input ratios ranged between 4.0 × 10^-6 and 2.3 × 10^-3 % for panning III and between 0.7 × 10^-6 and 2.3 × 10^-3 % for panning IV. After the final panning, 44 single clones of Fab phage were grown from each panning in 48-well plates and analyzed for reactivity with ch14.18 and 14G2a in ELISA. After restriction digestion with frequent-cutting BsrG I, indicating some genetic variety, 10 positive clones from each of the four experiments were sequenced.

**Characterization of selected clones**

Sequencing revealed at least 14 clones with different DNA sequences among the 40 picked clones. One clone lacked a L chain and was not further analyzed. Importantly, one clone (GK8) was isolated as often as 19 times. All 13 different clones proved to have anti-idiotypic properties by binding to both ch14.18 and 14G2a in

![FIGURE 1.](image-url) Patients’ sera were tested for the presence of anti-idiotypic Abs following anti-GD2 therapy with ch14.18. Serum samples had been drawn on day 30 after the fifth ch14.18 therapy treatment of neuroblastoma patient 1, 15 days after the fourth ch14.18 treatment of patient 6, and 21 days after the fifth therapy treatment of neuroblastoma patient 8. Serum from two healthy controls, control 1 and control 2, served as a negative control, and an anti-mouse IgG served as a positive control (ps. and pos.). a, Microtiter plates were coated with 14G2a. Only sera of patients that showed reactivity in a preliminary assay (not shown) were tested here again for antivariable region reactivity, with 14G2a. Only sera of patients that showed reactivity in a preliminary assessment on OKT3-coated plates. Only at high IgG concentrations did framework reactivity of the serum of patient 1 was excluded by binding assessment on OKT3-coated plates. Only at high IgG concentrations did the serum of patient 1 demonstrate weak binding.

![FIGURE 2.](image-url) Depicted is the percentage of binding inhibition as determined by B/Bo, where B is absorbance with inhibitor and Bo is absorbance without inhibitor. Bo was set at measurement endpoint OD of 2.5. Serum from patient 1 completely inhibited binding of ch14.18 to GD2. Serum from a healthy donor served as a negative control. Serum from a neuroblastoma patient, who had been treated with ch14.18 at another institution and therefore was not included in this study, showed very little binding inhibition.

![Graph A](image-url)

**Graph A.** Percentage of binding inhibition of ch14.18 to GD2 by serum of patient 1 and two controls (a neuroblastoma patient and a negative (Neg.) control). Depicted is the percentage of binding inhibition as determined by B/Bo, where B is absorbance with inhibitor and Bo is absorbance without inhibitor. Bo was set at measurement endpoint OD of 2.5. Serum from patient 1 completely inhibited binding of ch14.18 to GD2. Serum from a healthy donor served as a negative control. Serum from a neuroblastoma patient, who had been treated with ch14.18 at another institution and therefore was not included in this study, showed very little binding inhibition.

![Graph B](image-url)

**Graph B.** Percentage of binding inhibition of ch14.18 to GD2 by serum of patient 1 and two controls (a neuroblastoma patient and a negative (Neg.) control). Depicted is the percentage of binding inhibition as determined by B/Bo, where B is absorbance with inhibitor and Bo is absorbance without inhibitor. Bo was set at measurement endpoint OD of 2.5. Serum from patient 1 completely inhibited binding of ch14.18 to GD2. Serum from a healthy donor served as a negative control. Serum from a neuroblastoma patient, who had been treated with ch14.18 at another institution and therefore was not included in this study, showed very little binding inhibition.
repetitive ELISAs (Fig. 3). Three clones (T14–10, TCH-1, and TCH-2) provided problems in sequence analysis and are not listed in the tables and sequence alignments (Fig. 4). Use of H and L chains and amino acid sequences of CDRs varied greatly. Amino acid sequences revealed sequence identities between 26.8 and 100% among the H chains of the selected clones (Fig. 4a) and between 28.5 and 100% among the L chains of the selected clones (Fig. 4b). Identical sequences are only shown once in the figures.

Table I shows the corresponding germline gene origins for the different Ab chain sequences. Identities to different VH germ-line genes were between 94.9 and 100%. Three different VH families were used. Seven of 10 different clones belonged to the VH3, 2 of 10 to the VH1, and 1 of 10 to the VH4 family. Fifty percent of all VH sequences originated from the most common 3-30 (3 of 10 clones) or 3-23 (2 of 10 clones) germline gene loci. DH and JH sequences could be aligned to all 10 clones. However, as indicated in Table I, several D<sub>H</sub> sequences had to be aligned manually because of weak homology scores from V-BASE, leaving open several possibilities. J<sub>H</sub>4b was found in 8 of 10 clones.

Ab L chains included Va1, Va6, Ve1, and Ve3 chains. Five of 10 clones had a λ L chain predominantly belonging to the L1 family (4 of 10). The κ L chains used belonged to the Kl (3 of 10) and K3 (2 of 10) L chain families with almost equal spread. Identities to their putative V<sub>L</sub> germline genes ranged between 93.90 and 99.60%.

Mutation rates of selected clones in relation to corresponding V<sub>H</sub>1, germline sequences are shown in Table II. Remarkably, in the framework region not 1 of 10 clones showed a replacement-to-silent mutation (R:S) ratio of >2.9 for the H chain, whereas 6 of 10 did for the L chain. With regard to CDRs 1 and 2, 6 of 10 clones revealed an R:S ratio of >2.9 for the H chain, and 6 of 10 did for the L chain CDRs. CDRs 1 and 2 contained no replacement mutations in 3 of 10 H chains and 1 of 10 L chains. An R:S ratio of >2.9 in 60% of all H chain CDRs and 60% of the L chain CDRs may be indicative of Ag-driven Ab selection by ch14.18. It should be noted that H chain CDR3, which normally participates strongest in Ag binding, cannot be included in this analysis because it is composed of irregular VDJ, P/N nucleotide addition, irregular frame shifts, and inverse D combinations that occur before Ag selection. The results presented above relate to 10 different clones among 36 evaluable, anti-idiotypic Ab clones. Considering that several clones appeared as frequently as 19 times among the 36 analyzed clones, their significance is clearly increased.

**Anti-idiotypic properties of GK2 and GK8**

Anti-idiotypic binding properties of both Fabs and the corresponding Fab phages of GK2 and GK8 following large scale production and purification are depicted in Western blot analyses (Fig. 5, a and b). GK2 and GK8, both primarily selected by biopanning on murine 14G2a, also bind specifically to chimeric ch14.18 and, thus, are anti-idiotypic Abs.

Secondly, inhibition of ch14.18 binding to GD2 could be demonstrated for the isolated Fabs. Concentrations as low as 1.6 µg of GK2 Fab and 0.8 µg of GK8 Fab were able to inhibit binding of ch14.18 to GD2 by 70% or completely, respectively. An anti-BSA Fab, used as a control, showed no competition (Fig. 6). This confirmed that GK2 and GK8 are anti-idiotypic Abs of the type Ab2β or Ab3γ.

GK8 recognized and bound to the same epitope as the natural anti-idiotypic serum Abs of patient 1 directed at a concentration of 2.8 µg. GK8 Fab suppressed binding of diluted (1/20,000) patient serum to 14G2a by 84%. GK2 Fab completely lacked this capacity (Fig. 7).

Proof that they are true internal image Abs (Ab2β) of GD2 was found for GK8 Fab and, to a much lesser extent, for GK2 Fab when they induced anti-GD2 Abs (Ab3) in the xenogeneic rabbit system. Preimmune rabbit sera were tested for positive reactivity with GK2 and GK8 Fabs and the corresponding Fab phages (data not shown) and for positive GD2 binding to preclude presence of Ab3 before vaccination. All rabbit sera demonstrated a very strong reactivity to their respective vaccine in ELISA 42 days after immunization, including the anti-BSA control (not shown).

After immunization, rabbits of group 1, immunized with GK2 Fab and GK8 Fab, demonstrated an increase of anti-GD2 titers between day 0 and day 63 after initial vaccination, which was more pronounced for GK8 Fab (Fig. 8a). Samples were adjusted for their IgG content to account for variable IgG levels in different rabbits before and after immunization. Vaccination of rabbits of group 2, which received GK2 and GK8 Fab phage, again demonstrated positive anti-GD2 responses (Ab3) after treatment. In contrast, a control immunized with the anti-BSA Fab phage remained negative on GD2. Samples of group 2 were only available until day 42 after initial immunization (Fig. 8b). In summary, GK2 and GK8 were able to induce an anti-GD2 Ab response in both experimental settings, which was very weak for GD2 but strong for GK8. Thus, at least GK8 can be termed a true Ab2β.

As expected, the use of human Fab as a vaccine in rabbits also elicited immune responses against the framework region of the injected human fragment. The background of interfering Abs could be reduced by preincubation with IVIG. Without preincubation, a 2.98-fold increase for GD2-binding Abs against GK8 Fab phage was observed upon comparison of prevaccination and postvaccination samples. Preincubation with IVIG resulted in a 4.06-fold increase of specific GD2-binding Abs (data not shown).

**Discussion**

Phage display technology enabled the cloning of a repertoire of anti-idiotypic Abs (Ab2) from B lymphocytes of a tumor patient after passive immunotherapy with chimeric anti-GD2 Ab ch14.18, designated Ab1 in this case. Upon repetitive selection of Fab phage...
display libraries on the target Ag ch14.18 and its murine relative 14G2a, positive binders were enriched. The selected Ab2 clones GK2 and GK8 as well as another 38 Fab phage clones demonstrated strong reactivity with both ch14.18 and 14G2a. This was repeatedly confirmed (Fig. 3). Specificity and selectivity of binding was confirmed in Western blot analysis (Fig. 5, a and b). Both anti-idiotypic clones inhibited binding of ch14.18 to the tumor-associated Ag GD2 (Fig. 6). Surprisingly, GK8 Fab alone was able to inhibit binding of all of the anti-idiotypic Abs of patient 1 by 84%, suggesting that it is identical with the majority of the patient’s original anti-idiotypic Abs (Fig. 7) (66). Immunization of rabbits with either soluble GK2 or GK8 Fab or their Fab phages produced a continuous rise in Ab3 serum levels (Ab3) in these animals, as indicated by increased GD2 binding (Fig. 8, a and b). The cloned human anti-idiotypic Fab GK8 may be suitable as a tumor vaccine.

FIGURE 4. Alignment of 10 different, complete amino acid (aa) sequences. Shaded areas mark amino acid identities among the clones. a, Alignment of amino acid sequences of Ab H chain variable regions. According to the Kabat nomenclature (91) CDR1, CDR2, and CDR3 correspond to the amino acid sequences 32–37, 52–68, and 101–122, respectively, in this figure. b, Alignment of amino acid sequences of Ab L chain variable regions. According to the Kabat nomenclature (91) CDR1, CDR2, and CDR3 correspond to the amino acid sequences 22–34, 50–56, and 91–101, respectively, in this figure. GenBank accession no. AM259131-AM259150.
This approach allowed the characterization of the molecular origin of totally human anti-idiotypic Abs cloned from a previously treated neuroblastoma patient. To the best of our knowledge, this is the first time that numerous totally human Ab2s have been directly cloned from a tumor patient’s B cell repertoire, permitting deeper insight into the Ig gene usage within the anti-idiotypic Ab repertoire.

Screening neuroblastoma patients for Ab2-serum levels after ch14.18 treatment to choose a suitable B cell donor demonstrated that, in our single center study, among the 65 serum samples of nine patients analyzed, only three patients produced a significant immune response against the variable region of ch14.18 (Ab2). These three patients are still alive without signs of disease for 4.5–7 years and have been off treatment for 4–5.5 years. Regardless of Ab2 serum reactivity, no Ab3 could be measured in patients’ sera available for testing. However, although first Ab3 responses had been reported as early as 6 mo after initial Ab1-treatment, time intervals of 12 mo following initial ch14.18 treatment (allowing for regular blood draws according to therapy protocol NB97 of the Society for Pediatric Oncology and Hematology in our case) may still have been too short according to therapy protocol NB97 of the Society for Pediatric Oncology and Hematology in our case) may still have been too short for regular blood draws according to therapy protocol NB97 of the Society for Pediatric Oncology and Hematology in our case) may still have been too short for the generation of a natural immune response needed for the activation of the idiotypic network. Applying 47 courses of ch14.18 treatment to nine patients, we rarely observed any side effects, in agreement with the overall results of our NB97 multicenter study (69).

Discrepancies between the studies of Yu et al. (14), Cheung et al. (30), and Ozkaynak et al. (68) and our results are striking in respect to anti-GD2 immune responses. Whereas they had a high frequency of immune responses to ch14.18 and 3F8, we only detected an immune response to ch14.18 in three of nine patients (14, 20, 68). Neither of the above studies asked for precautionary routine administration of corticosteroids to preclude anaphylactic reaction to mAb ch14.18 as did our protocol NB97 (14, 54, 68). Corticosteroid premedication at least partially counteracts the initiation of a natural immune response needed for the activation of the idiotypic network. Applying 47 courses of ch14.18 treatment to nine patients, we rarely observed any side effects, in agreement with the overall results of our NB97 multicenter study (69). Interestingly, patient 1, who was the only one in our patient group not pretreated with corticosteroids and who suffered from ch14.18 side effects such as severe hyperthermia, pain, and transient renal tubulopathy during/following his first course of ch14.18 therapy, developed significant Ab2 levels against ch14.18 and is still in remission (Fig. 1a).

Anti-idiotypic Abs were and are used as surrogate tumor-associated Ags in numerous vaccination trials, producing clinical remissions and confirming the idiotypic network. Until now, for the most part murine and other xenogeneic anti-idiotypic Abs, partially humanized or genetically modified, were cloned for tumor immunotherapies. Applying 47 courses of ch14.18 treatment to nine patients, we rarely observed any side effects, in agreement with the overall results of our NB97 multicenter study (69). The latter results parallel those of our rabbit vaccination experiments, which showed significant immune responses toward the idiotypic network. Applying 47 courses of ch14.18 treatment to nine patients, we rarely observed any side effects, in agreement with the overall results of our NB97 multicenter study (69). The latter results parallel those of our rabbit vaccination experiments, which showed significant immune responses toward the idiotypic network. Applying 47 courses of ch14.18 treatment to nine patients, we rarely observed any side effects, in agreement with the overall results of our NB97 multicenter study (69). The latter results parallel those of our rabbit vaccination experiments, which showed significant immune responses toward the idiotypic network.
with pooled human IgG. Similar adsorption experiments demonstrated an Ab3 response, i.e., positive GD2 binding in 40 of 47 melanoma patients treated with 1A7 (36).

When human anti-idiotypic Ab 105AD7, produced by the fusion of a human/mouse heteromyeloma and B cells of a patient treated with 4791T/36 (a mouse Ab directed against gp72), was applied to osteosarcoma patients, 11 of 28 patients had an Ab3 response (38, 71). Fusion cell lines of human/mouse heteromyelomas, however, frequently are not stable and contain murine glycosylation (72–75). Use of a completely human vaccine in the human system should therefore be advantageous and even more effective by overcoming problems of nonspecific immunoreactivity. A possible alternative may also be peptide mimotopes of GD2 (76, 77).

Goletz et al. (78) described the selection of large diversities of single-chain variable fragment (scFv) anti-idiotypic Abs from three large naive human phagemid libraries (Griffin I and Tomlinson I + J libraries). They accomplished an increase of anti-idiotypic scFv binders by improving elution and selection procedures of scFv. For the glycin elution, which was also used in our work, their yield of specifically binding anti-idiotypic scFv was 5

<table>
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<tr>
<th>Table II. Mutations rates in 14G2a/Ch14.18 selected VH and VL</th>
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<td>Clones</td>
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a No./Total means number of mutations/number of all bases in the region.

b R/S represents number of replacement/silent mutations.

c Ratio stands for R/S ratio.
d denotes infinity, although mathematically not correct.

Six of ten (60%) different clones showed an R/S ratio of >2.9 for CDRs 1 and 2 of their H chains, and six of ten (60%) did so for CDRs of their L chains. Taking into account that some clones appeared repeatedly, 32 of 36 (89%) clones showed somatic re-placement mutations in CDRs of VH regions, and 11 of 36 (31%) did so for CDRs of their VL regions.

This result is interesting, because earlier studies on bone marrow transplant (BMT) recipients had demonstrated that rearrangements in BMT recipients exhibited far fewer somatic mutations than did rearrangements from healthy subjects (86, 87). Although our B cell donor did not undergo BMT, intensive chemotherapy is also known to destroy lymphoid tissue. Because the failure to accumulate somatic mutations in rearranged VH genes is consistent with a maturational arrest at a very late state of B cell differentiation, and because somatic mutations and affinity maturations are thought to take place in lymph node germinal centers, it is a popular hypothesis that the failure of germinal center processes prevents normal accumulation of somatic mutations following immunization in BMT recipients (86, 87). But, unlike studies in BMT recipients, Ab clones picked from our “immunized” library from a heavily pretreated neuroblastoma patient exhibit a proportion of somatic mutations, comparable to what we and others found in B cell li-

braries of healthy subjects (60, 87). In this study, 30% of somatic mutations were detected in the B cell population of these subjects, divided into 10% of such mutations in preimmune B cells, 70% in Ag-stimulated B cells, and 20% IgM mutations. In contrast, only 1–10% of somatic mutations were found in B cells of BMT recipients (87). In summary, the immunological capacity of our patient to respond to foreign Ags seems rather comparable to that of a healthy subject.

Further studies on BMT recipients and the reconstitution of the Ig VH repertoire were able to describe particular patterns of VH expression in healthy subjects and allografted/autografted patients, the latter mimicking ontogeny of the Ig repertoire (88). Although there is a bias due to PCR primer selection, the use of VH-genes in 10 different Ab2-clones in our current study was VH3 (70%) /H11022 VH1 (20%) /H11022 VH4 (10%). Accounting for the total numbers of clones changed the order to VH4 (53%) /H11022 VH3 (42%)/H11022 VH1.

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creases are suggestive of a recovering immunological status at a healthy subject, the ab-

(6%). Whereas the relative numbers in the first order resemble the normal numbers in the second order. In contrast to the anti-id antibody production, a control rabbit immunized with the anti-BSA Fab phage showed no increase in binding to GD2. The data are representatives of several independent assays with or without preadsorption on IgG that used anti-idiotype Abs, GK8, may be useful as a human GD2 surrogate tumor vaccine.

**Acknowledgments**

We thank Heike Lurch for excellent technical assistance, Prof. G. Gaedicke (Charité, Berlin, Germany) and Prof. A. Yu (University of California at San Diego, La Jolla, CA) for discussion, Prof. R. Handgretinger (St. Jude Children’s Hospital, Memphis, TN) for providing 14G2a, and Prof. C. Barbas (The Scripps Research Institute, La Jolla, CA) for providing the pComb3H vector. Moreover, we thank the personnel of wards 30, 39, and T23 Hem/Onc outpatient center (Charité Children’s Hospital) for their help in sampling patient sera.

**Disclosures**

M. M. Utenreuther-Fisher, J. A. Krüger, and P. Fischer have applied for a German patent, "Mittel humanen Ursprungs zur Vakzination gegen GD2-positive Tumore."