Immunogenicity of therapeutic antibodies
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Chapter 5

Neutralizing capacity of monoclonal and polyclonal anti-natalizumab antibodies: The immune response to antibody therapeutics preferentially targets the antigen-binding site


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To the Editor:

Many therapeutic monoclonal antibodies have been found to elicit an immune response to various degrees. Anti-drug antibodies (ADA) can diminish efficacy by neutralization or enhanced clearance of the therapeutics, and occasionally cause hypersensitivity reactions or other adverse events.1 Therapeutic antibodies are classified as chimeric, humanized or ‘fully human’, in order of increasing homology of the variable domains with human germ-line sequences, and thereby their expected decrease in immunogenicity.

Recently, our group investigated the antibody repertoire in patients with an immune response against any of four different anti-TNF therapeutic antibodies.2,3 For the human(ized) adalimumab, certolizumab and golimumab, >94% were found to bind the TNF binding site, demonstrating that these ADA are essentially all neutralizing. Strikingly, the antibody response to the chimeric infliximab, of which the entire variable domains are of mouse origin, was also >90% directed to the TNF binding site. These results prompted the question whether this restricted response is due to specific immunogenic properties of the anti-TNF binding site, or that the antigen binding site (or paratope) is an inherently immunodominant part of the antibody, irrespective of its target. If so, anti-drug antibodies to any therapeutic antibody might be expected to be predominantly neutralizing.

To shed light on this question, we investigated the antibody response to natalizumab, a humanized antibody that differs in target (α4-integrin), subclass (IgG4) and patient group (relapsing-remitting multiple sclerosis, RRMS) from anti-TNF therapeutics. Natalizumab contains several non-human germline determinants outside of its paratope (Figure 1A) and can thus theoretically elicit a broad non-neutralizing antibody response. In this study, we examined the neutralizing capacity of the anti-natalizumab response of RRMS patients.

We therefore first developed human monoclonal anti-natalizumab antibodies. Variable domain sequences of in total three natalizumab specific peripheral blood B cells were obtained from two patients (Table E1). These sequences were recombinantly expressed in HEK293F cells, resulting in three human monoclonal anti-natalizumab antibodies. Analysis of the clones using ImMunoGeneTics/V-QUEry and STandardization (IMGT/V-Quest)4 revealed a distinct V(D)J gene usage for each clone. Using surface plasmon resonance (SPR), the affinity of anti-natalizumab 1.1 and 2.1 was found to be moderate, whereas anti-natalizumab 2.2 had a high affinity (1x10⁻¹¹ mol/L; Figure E1, Table E2).

To investigate the natalizumab binding site for each clone relative to the others, the antibodies were tested in a competitive ELISA. In this assay, the binding of a labeled
clone was assessed in the presence of a competing non-labeled clone (Figure 1B). The results showed that the monoclonal antibodies all competed with each other for binding to natalizumab (Figure 1C), indicating that all three monoclonal antibodies bind to a restricted site on natalizumab.

We further examined whether the monoclonal antibodies could block the binding of natalizumab to its target α4-integrin. This was tested using fluorescently labeled natalizumab that binds α4-integrin expressed on Jurkat cells. Preincubation of natalizumab with any of the three monoclonal antibodies abrogated the binding of natalizumab to α4-integrin (Figure 1D,E). Similar results were found for the binding of exchanged (i.e. monovalent, see ‘IgG4 half-molecule exchange’ in Repository Text) natalizumab to α4-integrin (Figure E2). This indicates that all three monoclonal anti-natalizumab antibodies compete with α4-integrin for binding to natalizumab, and are thus all neutralizing.

Next, we investigated the neutralizing capacity of polyclonal anti-natalizumab antibodies from 15 ADA-positive patients. A competitive ELISA was set up in which α4-integrin blocks the paratope of natalizumab (Figure 2A). For all patients, the binding of ADA to natalizumab was reduced with increasing concentrations of α4-integrin (Figure 2B). With the highest amount of α4-integrin tested, the signal was reduced by at least 91% compared to the uninhibited signal (Figure 2C). This indicates that for all patients tested, at least 91% of all ADA to natalizumab are neutralizing.

We further analyzed which parts of natalizumab are targeted by the anti-natalizumab response using a similar competitive assay, but now using neutralizing, but less bulky, Fab fragments of monoclonal anti-natalizumab 2.2 to block the binding site of natalizumab (Figure 2D). Again, the binding of patient ADA to natalizumab could be reduced in a concentration-dependent manner, ultimately preventing at least 92% of ADA from binding natalizumab using this single monoclonal antibody (Figure 2E,F). This implies that the majority of ADA from RRMS patients binds determinants of natalizumab located in or in close proximity to the paratope.

The results from this present study on the anti-natalizumab response, in combination with our previous findings on the neutralizing capacity of ADA to anti-TNF therapeutics,2,3 indicates that the immune response preferentially targets the paratope of at least several classes of therapeutic antibodies. The antigen specificity of the drug seems irrelevant for this response. In addition, the non-human germline determinants outside of the paratope found in natalizumab, as well as those in the chimeric infliximab (and even in the other humanized and fully human anti-TNFs) apparently are less immunogenic. This
Figure 1. Monoclonal anti-natalizumab antibodies compete with each other and with α4-integrin for binding natalizumab. A) Crystal structure of natalizumab Fab (orange and purple) in complex with the α4 headpiece (green). Blue residues differ from human germ-line. Adapted from Yu et al. B) Schematic representation of the competitive ELISA. BT = biotinylated, NTZ = natalizumab. C) Monoclonal anti-natalizumab antibodies bind natalizumab and compete with each other. D) Fluorescently labeled Natalizumab (NTZ-488) binds α4-integrin expressed on Jurkat cells; neutralizing antibodies inhibit this binding. E) Left: Representative histogram of the ratio-dependent reduction of natalizumab binding to α4-integrin induced by monoclonal antibody 1.1. Right: Inhibition of natalizumab binding to cell surface α4-integrin by all three monoclonal antibodies determined by FACS. BT, Biotinylated; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; NTZ, natalizumab.
observation is also consistent with the lack of immune response towards mismatched allotypes of therapeutic antibodies. The paratope of therapeutic antibodies, but perhaps of any antibody, might be intrinsically more immunogenic than other determinants outside of the paratope. It is however unknown whether the paratope displays specific structural features that facilitates its potential role as epitope.

Figure 2. The majority of serum anti-natalizumab antibodies neutralize natalizumab. A,D) Schematic representation of the competitive ELISA. BT = biotinylated, NTZ = natalizumab. B,E) Increasing amounts of α4β1 (B) or anti-natalizumab 2.2 Fab (E) decrease ADA binding to natalizumab. Each line represents one patient. A selection of representative patients is shown. C,F) Percentage of AU (arbitrary units) inhibited by 15 µg of α4β1 (C) or anti-natalizumab 2.2 Fab (F). Each dot represents a single patient. Median and range for inhibition with α4β1 93% (91%-96%) and anti-natalizumab 2.2 Fab 97% (92%-99%), n=15.

For infliximab, attempts were made to more precisely locate immunogenic amino acid sequences using synthetic peptides. Homann et al. found reactivity of ADA-positive patients to four peptide sequences located in or in close proximity to the complementarity determining regions (CDRs), although two of these peptides were also recognized by healthy controls. By contrast, a study by Kosmac et al. showed no reactivity to synthetic peptides nor to denatured infliximab Fab, suggesting that anti-infliximab antibodies target conformational rather than linear epitopes. It therefore remains unclear which precise determinants on infliximab are targeted. For adalimumab, we demonstrated
that anti-adalimumab antibodies bind to multiple overlapping, but distinct epitopes in the paratope.³

Human(ized) and chimeric therapeutic antibodies all have fully human constant domains. Although there is little evidence for drug-induced antibody responses towards these regions, pre-existing antibodies such as rheumatoid factors (low-affinity IgM antibodies binding human IgG Fc) can be present. The available literature suggests that the presence of such antibodies generally has little consequences.⁶

Our findings on the neutralizing capacity of ADA to natalizumab are in line with a study of Calabresi et al.⁹ in which ADA to natalizumab were analyzed using an ELISA that measures all ADA and a cell-blocking assay that only measures neutralizing ADA. The strong correlation between the two assays suggested that most or all ADA to natalizumab were neutralizing.

In conclusion, this study shows that the majority of the polyclonal anti-natalizumab response in RRMS patients specifically targets the antigen binding site of natalizumab, and thereby extends the results previously obtained for anti-TNF antibodies.²,³ Although exceptions cannot be ruled out, these results suggest that the immune response towards antibody therapeutics may preferentially be directed towards the paratope of the drug, and will thus inherently neutralize the therapeutics.

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Conflict of interest
AV has received speaking fees from Teva. VUmc MS Center Amsterdam has received financial support for research activities from Bayer Schering Pharma, Biogen-Idec, GlaxoSmithKline, Merck Serono, Novartis, and Teva. JK has accepted speaker and consulting fees from Merck-Serono, TEVA, Biogen, Genzyme, Roche and Novartis. The VUmc MS Center Amsterdam has received financial support for research activities from Bayer Schering Pharma, Biogen, Glaxo Smith Kline, Merck Serono, Genzyme, Roche, Novartis, and Teva. GW has received a research grant from Pfizer and honoraria for lectures from Abbvie, Pfizer and UCB. TR has received honoraria for lectures from Pfizer, Abbvie, and Regeneron, and a research grant from Genmab.
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**Table E1.** Characteristics of human monoclonal anti-natalizumab antibodies. For each clone the isotype, V(D)J usage, number of mutations leading to amino acid replacements (R) or silent mutations (S) and κ/λ is described. Sequences were aligned using IMGT/V-QUEST database.¹

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**Table E2.** Estimated affinities of anti-natalizumab clones for natalizumab Fab’. Association and dissociation rates and Kd of anti-natalizumab to natalizumab Fab’ are calculated from SPR measurements.

<table>
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Figure E1. Sensorgram of natalizumab Fab’ binding to monoclonal anti-natalizumab antibodies. Monoclonal anti-natalizumab is bound to immobilized anti-human IgG. Association and dissociation of natalizumab Fab’ to the clones is shown. Three different concentrations (µg/ml, shown right) were used to estimate the Kd. Sensorgrams are made using SPR (surface plasmon resonance) analysis at 25°C, RU = Response Unit.
Figure E2. Monoclonal anti-natalizumab antibodies compete with α4-integrin for binding natalizumab. 
A) Schematic representation of the competition FACS experiment. Exchanged natalizumab binds α4-integrin expressed on Jurkat cells. Neutralizing antibodies inhibit this binding. B) Inhibition of exchanged natalizumab binding to cell surface α4-integrin by all three monoclonal antibodies determined by FACS. FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; NTZ, natalizumab.
MATERIALS AND METHODS

Patients
For the development of human monoclonal anti-natalizumab antibodies, EDTA blood was collected from two ADA-positive RRMS patients. For the competitive assays, serum samples of 7 patients with anti-natalizumab antibodies were selected from a prospective observational cohort previously described by Vennegoor et al.2 After obtaining informed consent, blood was taken from these patients. The study was approved by the ethics committee of the VU Medical Center, Amsterdam. In addition, anti-natalizumab antibody positive sera were obtained from our diagnostic department (n=8). Materials were used anonymously without any connection to clinical data. Anti-natalizumab positivity was measured using a previously described antigen binding test.3

Development of human monoclonal anti-natalizumab antibodies
Natalizumab specific B-cells were isolated essentially as described before, 4 with the modification that DAPI negative, anti-CD20-PerCP-Cy5.5 positive, anti-IgD-FITC negative (all from BD Biosciences) memory B-cells were selected, after which cells double positive for natalizumab F(ab)2-AF647 (Invitrogen, labeled according to the manufacturer’s instructions) and biotinylated natalizumab F(ab)2 combined with streptavidin-PE (BD Bioscience) were placed one cell per well in 96-well flat bottom plates. Determining the type of subclass and light chain of monoclonal anti-natalizumab antibodies, and production of the recombinant antibodies was done as described before.5

Natalizumab bridging ELISA
B cell supernatants and recombinant monoclonal anti-natalizumab antibodies were tested for anti-natalizumab specificity using a bridging ELISA as described before, with slight adjustments.4 In short, samples (diluted appropriately in PTG (PBS containing 0.2% gelatin (Merck, Germany) and 0.1% Tween-20) were added to a natalizumab coated plate (0.25 µg/ml in PBS), followed by detection with biotinylated natalizumab F(ab)2 (50 ng/ml).

Generation of F(ab)2 and Fab’ and Fab fragments
F(ab’)2 and Fab’ fragments were generated using pepsin, and subsequent reduction and alkylation with dithiothreitol and iodoacetamide respectively, essentially as described.6 Fab fragments were generated by papain digestion as described,7 with the exception that the enzyme:antibody ratio was 1:10.
**Surface plasmon resonance measurements**

Surface plasmon resonance measurements were performed using a Biacore T200 instrument (GE Healthcare) at 25°C. Mouse anti-human IgG (MH16-1, Sanquin) was immobilized at a concentration of 5 µg/ml in 10 mM sodium acetate, pH 5.0, at a flow rate of 10 µl/min on a CM5 sensor chip using N-hydroxysuccinimide/1-ethyl-3-(3dimethylaminopropyl)carbodiimide hydrochloride (NHS/EDC). Anti-natalizumab monoclonal antibodies were dissolved at 5 µg/ml in PBS pH 7.4, containing 0.05% Tween-20 (PBS-T), and passed through the cells at 15 µl/min yielding ca. 100-300 response units (RU) of bound antibody. Subsequently, binding of natalizumab Fab' (0.125 – 1.25 µg/ml) dissolved in PBS-T was measured at a flow rate of 15 µl/min. After each run, immobilized ligand was regenerated by removing bound analyte with 5 µl of 0.1 M phosphoric acid. Adsorptions obtained in the reference channel without bound anti-natalizumab antibody was subtracted from the adsorptions in the other cells. Association and dissociation kinetics were fitted using the models provided with the Biacore analysis software.

**Competitive ELISA with mAbs as inhibitor**

A 96-well flatbottom MaxiSorp plate was coated with 0.25 µg/ml natalizumab in PBS and incubated overnight. Plates were washed with PBS-T. Biotinylated anti-natalizumab was preincubated at 0.15 µg/ml in PTG together with unlabeled anti-natalizumab at a final concentration of 1 or 5 µg/ml and subsequently added to the plate. After 1 hour incubation, plates were washed and incubated for 25 minutes with streptavidin-poly HRP (horseradish peroxidase, Sanquin, The Netherlands) diluted in PTG, after which plates were washed again and developed using substrate solution. Samples were measured at 450nm and 540nm.

**IgG4 half-molecule exchange**

IgG4 antibodies have the property to exchange half-molecules (i.e. one heavy chain and its attached light chain) with other IgG4 molecules. This half-molecule exchange occurs under physiological conditions in patients and healthy people. Natalizumab is an IgG4 antibody and is able to exchange with autologous IgG4, thus becoming monovalent.8 To create monovalent fluorescently-labeled natalizumab, natalizumab labeled with DyLight488 (natalizumab-AF488, Invitrogen, labeled according to the manufacturer’s instructions) was incubated with a 100-fold excess of anti-adalimumab IgG4 in the presence of 1mM GSH (reduced glutathione) at 37°C as described previously.3 After 24h the reaction was stopped by adding iodoacetamide to a concentration of 1mM. A bridging ELISA was used to check exchange of natalizumab: exchanged natalizumab is unable to form a bridge between coat and detection antibody.
Competitive FACS experiments

Jurkat cells (a kind gift of M. Schreier (Sandoz, Basel, Switzerland)) were washed with PBA (PBS+0.1% BSA (Sigma-Aldrich)). Natalizumab-AF488 was preincubated for 15 minutes with anti-natalizumab in a ratio of 1:1, 1:5 or 1:25 (natalizumab-AF488 (1 µg/ml): anti-natalizumab) and subsequently added to the cells in a 20x dilution. In addition, natalizumab-AF488 without inhibiting anti-natalizumab was added to the cells in a 20x dilution. A similar setup was performed with exchanged (i.e. monovalent) natalizumab-AF488. Binding of natalizumab-AF488 to Jurkat cells was determined using the Beckton Dickinson LSR Fortessa (BD Biosciences) and analyzed with FACS DIVA software (BD Biosciences) and FlowJo software (v7.6.2; Treestar, Ashland, Ore).

Competitive ELISA with α4β1 or anti-natalizumab 2.2 Fab as inhibitor

First, patient sera were titrated to determine the optimal serum dilution (out of plateau) for every patient. Next, 96-well flatbottom MaxiSorp plates were coated with 31 ng/ml natalizumab in PBS and incubated overnight. For each sample, 12 wells were incubated with the optimal serum dilution (diluted in PTG). The standard curve consisted of a titration of monoclonal anti-natalizumab 2.2. Samples were incubated for 1 hour. Biotinylated natalizumab (5 ng/ml) was pre-incubated with a titration of the inhibitor (anti-natalizumab 2.2 Fab (generated using papain) or α4β1 (R&D Systems), both ranging from 0 – 15 µg/ml) and subsequently added to each patient serum. For the standard curve biotinylated natalizumab without inhibitor was used. After 1 hour incubation, plates were washed and incubated with streptavidin-poly HRP diluted in PTG for 25 minutes. Plates were washed again and developed using substrate solution as described above. Samples were measured at 450nm and 540nm. OD was converted into arbitrary units (AU) using the standard curve. Percentage of inhibition was calculated by dividing the AU of inhibited samples by the AU of non-inhibited samples.
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