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The Effect of Induced Antibodies with Respect to Neutralization, Clearance Rate and Functional Activity in a Rabbit/Infliximab Model

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Abstract. *Background: Therapeutic antibodies are a developing field for treatment of an expanding number of inflammatory diseases, including Crohn's disease. Treatment with monoclonal antibodies is frequently hampered by development of anti-drug antibodies (ADAs) that may compromise the treatment. Materials and Methods: We addressed this issue in a rabbit model of treatment with the anti-tumor-necrosis factor alpha (TNFα) antibody, infliximab (IFX). We developed an inhibition ELISA to selectively measure absolute concentrations of neutralizing antibodies and another ELISA for measuring the concentration of functional IFX in the circulation. Results: We found that the concentration of functional IFX was inversely proportional to the concentration of neutralizing antibodies. Conclusion: Administration of IFX to rabbits showed diversity in immune responses/tolerance toward IFX, corresponding to responses observed in patients. The applied assay technology is easily adapted to human plasma samples and/or other therapeutic antibodies, including fully humanized antibodies, for which immunogenicity also is observed.*

Therapeutic monoclonal antibodies or fusion proteins hereof are increasingly used for treatment of diseases. Unfortunately, such therapies are frequently hampered by immunogenic responses in the patients and the development of ADAs (anti-drug antibodies) of which some may directly inhibit the drug and/or increase its disappearance rate. This raises several important questions, including whether it is possible to personalize treatment, in advance, by predicting which patients possess or will develop ADAs and when a treatment should be stopped or replaced.

Infliximab (IFX) is a therapeutic monoclonal antibody against tumor necrosis factor alpha (TNFα). Efficacy in introducing and maintaining remission in inflammatory bowel disease, *i.e.* Crohn's disease (1-4), ulcerative colitis (5-7), rheumatoid arthritis, ankylosing spondylitis, psoriasis and psoriatic arthritis, is well-documented. Studies have shown that one third of patients with inflammatory bowel disease in IFX treatment are primary non-responders and one third are secondary non-responders. Within the primary non-responders, IFX treatment is initially and wholly inefficacious, whereas secondary non-responders exhibit a gradual loss of effect after an initially successful response to treatment (2, 3, 6, 8). IFX is a chimeric human-mouse immunoglobulin G1 (IgG1) monoclonal antibody produced in mouse hybridoma cells by recombinant DNA technology. It is composed of human constant regions and mouse variable regions, consisting of 75% human peptide sequences and 25% mouse peptide sequences (9, 10) (Figure 1). The formation of antibodies to anti-TNFα treatment is well-described (11-13). Antibodies to IFX have been associated with increased risk of infusion reactions but the relationship to lack of response or loss of response is more uncertain (14, 15). Concomitant immunosuppression therapy is known to reduce the levels of anti-IFX antibodies (16-18).

Up to 94% of healthy blood donors possess antibodies against mouse IgG. Some of these antibodies react directly with the mouse-derived antigen-binding fragment (Fab) of

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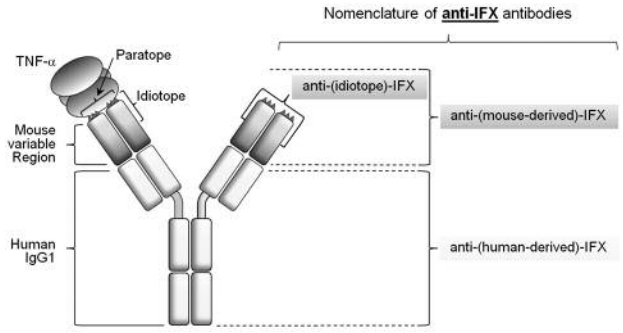

Figure 1. *Composition of infliximab (IFX) and definition of host anti-IFX antibody reactivity. IFX is a chimeric anti-TNFα antibody (IgG1) composed of mouse-derived variable regions and human-derived constant regions. The paratope constitutes the part of the variable region, which directly interacts with TNFα, while the idiotope represents the unique set of antigenic determinants of the variable portion of IFX. Host immune responses and development of anti-IFX (anti-drug-antibodies) may be categorized into three categories: anti-(human-derived)-IFX antibodies, anti-(mouse-derived-IFX) antibodies and anti-idiotypic antibodies of which the latter category is a subgroup of anti-(mouse-derived-IFX) antibodies.*

IFX and rituximab. It has been suggested that bovine antibodies, in certain vaccines, induce the production of anti-bovine-IgG antibodies (19) and that some of these antibodies cross-react with the mouse components in IFX and rituximab. The presence of anti-IFX-Fab-reactive IgG antibodies has been correlated with decreased long-term efficacy and safety issues in a retrospective analysis. This finding was only confirmed for Crohn's disease and not for ulcerative colitis (20). Hence, the clinical relevance of this finding is still uncertain and needs to be clarified.

The present study focuses on the level of circulating, neutralizing antibodies, *e.g.* anti-idiotype or antibodies resulting in steric hindrance of interaction with TNFα, and the anti-IFX-antibody-dependent disappearance rate of functional IFX. Reliable assessment of immunogenicity and half-life of anti-TNFα and other biologicals could be used to optimize dose regimens and prevent prolonged use of inadequate therapy. It could also assist clinicians in deciding when to change treatment strategy. With the introduction of "biosimilars", in the wake of an expired patent, one could expect a future rise in the use of biologics, partly due to their increasing affordability. Thus, the treatment with biological drugs is likely to remain a huge economic burden and better insight into functional half-life and immunogenicity could be of great value, both in relation to cost and personalized treatment.

The animal model and *in vitro* methods developed within the present work can be applied to analyze neutralizing ADAs and disappearance rate of an administered therapeutic antibody. We have used IFX, targeting TNFα, as a model antibody, but the methodology may be applied for other therapeutic antibodies as well. We studied drug functionality and neutralizing host antibodies in order to introduce methods that could be used as predictive technologies, hopefully directly applicable in clinically relevant human studies.

Materials and Methods

Buffers, reagents and materials. Unless otherwise stated, reagents were obtained from Sigma-Aldrich, Broendby, Denmark. The used buffers comprised: coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), PBS (1.45 mM NaH_2PO_4, 6.46 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 8.0), TBS (20 mM Tris, 125 mM NaCl, pH 7.4), ELISA washing buffer (TBS, 2 mM $CaCl_2$, 0.05% Emulfogen (polyoxyethylene 10 tridecyl ether)), ELISA sample buffer (TBS, 2 mM $CaCl_2$, 0.05% Emulfogen, 0.1% BSA), substrate buffer (50 mM citric acid, titrated to pH 5.0 with Na_2HPO_4). o-Phenylenediamine (OPD) was obtained from KemEnTech, Taastrup, Denmark. Horseradish peroxidase (HRP)-conjugated streptavidin was obtained from Invitrogen/ Fischer Scientific, Slangerup, Denmark. HRP-labelled swine anti-rabbit IgG was obtained from Dako, Glostrup, Denmark. CNBr-activated Sepharose was obtained from GE Healthcare, Brondby, Denmark. HRP-labelled rabbit anti-human IgG was obtained from Dako. IFX was obtained as "Remicade" from Schering-Plough/Merck & Co, Ballerup, Denmark. An illustration of IFX composition with idiotope, mouse- and human-derived domains is shown in Figure 1, together with an illustration of the reactivities of rabbit anti-IFX antibodies: anti-(human-derived)-IFX, anti-(mouse-derived)-IFX and anti-(idiotope)-IFX. TNFα was obtained from ProSpec-Tany TechnoGene Ltd, Ness Ziona, Israel.

Labeling of TNFα with biotin. TNFα was labeled with biotin using N-Hydroxysuccinimide (NHS)-biotin at a ratio of 1:15 according to the manufacturer's recommendation (Sigma-Aldrich).

Immunization and blood sampling - Animals, housing and ethical approval. Four female New Zealand White rabbits (NZW) were obtained from HB Lidköpings Kaninfarm (Lidköping, Sweden) at approximately 12 weeks of age (body weight=3 kg). On arrival they were pair-housed in a Scanbur EC2 system (Scanbur Technology A/S, Karlslunde, Denmark) on straw/hay bedding (Brogaarden, Gentofte, Denmark), provided with bite blocks (Tapvei, Kortteinen, Finland) and a plastic shelter (Lillico, Horley, UK). Feed, provided ad libitum, consisted of a complete muesli ("Chudleys Rabbit Royale"; Dodson & Horrell Ltd, Islip, UK), supplemented with lettuce, carrots and other vegetables (Grøntgrossisten, Valby, Denmark) and tap water. The animals were kept on an automated light-dark cycle (12 h/12 h, with 30 min of "twilight" at transitions); ambient temperature (15-21˚C) and humidity (55%) was maintained through 17 h^{-1} air changes on a room-level. The study was approved by the Danish Animal Experiments Inspectorate (carried out under license number 2012-15-2934-00077). All of the procedures were performed in accordance with the EU directive 2010/63/EU in a fully Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility and under the supervision of a local animal welfare committee.

Table I. *Administration of infliximab (IFX) and blood sampling*.*

Injec. #	1st					2nd		3rd	4th				End
Day	0_0h	0_2h	1	3	7	14_0h	14_2h	28	42_0h	42_2h	43	46	54
Injection	+					+		+	+				
Blood sampling	+	+	+	+	+	+	+		+	+	+	+	+

*Intervals for all *i.v.* and *s.c.* immunized rabbits. 0 h and 2 h indicate that blood sampling is performed before and 2 h after IFX administration, respectively. At day 54 the rabbits were euthanized.

Procedures. IFX was administered to four rabbits parenterally: Two rabbits received intravenously (*i.v.*) administrations through the ear vein (1.5 ml of 10 mg/ml IFX infused slowly, corresponding to a dose of approximately 5 mg/kg bodyweight), mimicking the standard protocol for dosing human patients, and two rabbits received subcutaneously (*s.c.*) immunizations (100 μg/rabbit of IFX deposited as four boli; total volume 200 μl of IFX solution mixed 1:1 with Freund's incomplete adjuvant (Statens serum Institut, Copenhagen, Denmark)). Subsequent administrations followed the schedule given in Table I, corresponding to a standard program for IFX treatment in humans. Blood was sampled, pre and post administration, from the ear veins at the time points provided in Table I. Blood was collected in EDTA-coated tubes and plasma was isolated by centrifugation and stored at –20˚C until analysis.

ELISA – general procedure. Unless otherwise stated, the following general ELISA procedure and materials were used in all assays. MaxiSorp 96-well plates were obtained from Nunc A/S, Roskilde, Denmark. Reagents were immobilized onto the plates in coating buffer overnight at 4˚C. Plates were washed four times in washing buffer between each step in the assay. After coating, wells were blocked in washing buffer for 15 min and samples were subsequently incubated in sample buffer at room temperature. HRP-labeled antibodies (2 hours at room temperature) or biotin-labeled TNFα in combination with HRP-conjugated streptavidin (2 hours and 30 min, respectively, at room temperature) were used for detection. Plates were developed using OPD (0.5 mg/ml) in substrate buffer with 0.03% of H_2O_2, according to the manufacturer's recommendations (Kem-En-Tec). Development was stopped with 1 M H_2SO_4 and plates were read at 490 nm with 650 nm as reference wavelength.

Specific ELISAs - Immune response following IFX administration: Measurement of titers of anti-IFX antibodies. The relative concentration of anti-IFX antibodies was estimated in plasma from all four rabbits (2 × *s.c.* and 2 × *i.v.*) after the rabbits were euthanized. Plates were coated with IFX (0.2 μg/ml) and incubated with two-fold dilutions of rabbit plasma, beginning at 1:1000, for 2 hours. Bound anti-IFX antibodies were detected using HRP-labeled swine anti-rabbit IgG and developed with OPD. To estimate the absolute concentrations of anti-IFX in plasma samples obtained during the period of IFX administration (Table I), plasma samples were diluted between 1:3,000 to 1:50,000 and analyzed using a purified pool of anti-IFX antibodies as standard (100-1 ng/ml) (described below).

Measurement of neutralizing anti-IFX antibodies: anti-(mouse-derived)-IFX antibodies. The relative concentration of anti-IFX antibodies with inhibiting effects on the interaction between IFX and TNFα was measured in plasma from all four rabbits (2 × *s.c.* and 2 × *i.v.*) after the rabbits were euthanized. Plates were coated with IFX (2 μg/ml) and subsequently incubated with 2-fold dilutions of rabbit plasma, beginning at 1:50, for 2 h. After wash, biotin-labeled TNFα (0.2 μg/ml) was incubated for 2 hours, subsequently detected using HRP-conjugated streptavidin (0.1 μg/ml) and developed with OPD. To estimate the absolute concentrations of inhibiting anti-IFX in plasma samples obtained during the period of IFX administration (Table I), plasma samples were diluted 1:10 to 1:3,000 and analyzed using a purified pool of inhibiting anti-(mouse-derived)-IFX antibodies as standard (600-5 ng/ml) (described below).

Detection of functional IFX in plasma. The disappearance of functional IFX in rabbit plasma over time after IFX administration was analyzed for all four rabbits. Plates were coated with streptavidin (5 μg/ml, Sigma) and incubated with 0.2 μg/ml biotin-labelled TNFα for 1 hour and washed. Plasma samples were diluted 1:10 or 1:1000 and incubated for 2 hours in wells and then washed. Bound IFX was detected using HRP-labeled rabbit anti-human IgG (2 μg/ml, Dako) and developed with OPD. An IFX standard (250-0.5 ng/ml), diluted in 10% or 0.1% normal rabbit serum, was used to estimate the concentrations of functional IFX in the samples.

Immuno-specific affinity chromatography. These techniques were used to isolate anti-IFX Ig from EDTA plasma and to deplete purified anti-IFX Ig for anti-(human-derived)-IFX antibodies. The final preparation of anti-(mouse-derived)-IFX antibodies was used as standards for absolute measurements of neutralizing antibodies (above).

Purification of rabbit anti-IFX antibodies. CNBr-activated Sepharose 4B was coupled with IFX (7 mg IFX/ml Sepharose) according to the manufacture's recommendations and the following fractionations were carried out on a ÄKTA FPLC system (GE Healthcare). Plasma obtained after euthanasia of one of the *s.c.* administrated rabbits (*S.C.1*) was diluted 1:1 with washing buffer (PBS, pH 7.4) and applied to the column. Bound anti-IFX was eluted using 0.5% citric acid, pH 2.5 and the eluted fractions were neutralized with 1 M Tris pH 8.2. The presence of anti-IFX antibodies in the eluted fractions was analyzed by ELISA as described above (Figure 2). A high purity of the antibodies in the anti-IFX fractions was confirmed by SDS-PAGE and Coomassie Brilliant blue staining (Figure 2).

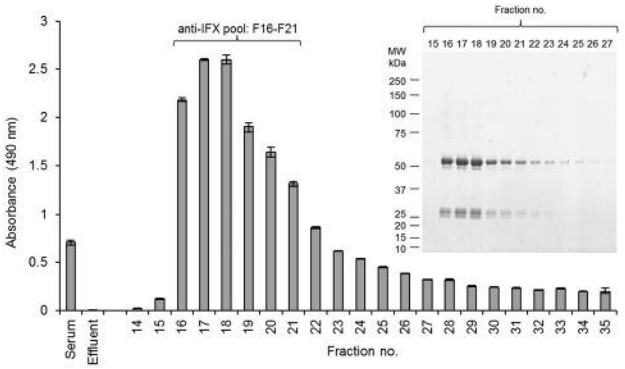

Figure 2. *Purification of anti-infliximab (anti-IFX) antibodies. Anti-IFX antibodies were isolated by immunoaffinity chromatography using a column conjugated with IFX and bound antibodies were eluted by lowering the pH in fractions 14-35. Purity of fractions was assessed by SDS-PAGE and Coomassie blue staining. Plasma from all three responding rabbits (s.c.1, s.c.2, i.v.2) was enriched for anti-IFX; the results shown derive from enrichment of s.c.1.*

Depletion of anti-(human-derived)-IFX antibodies. Affinity purified rabbit anti-IFX antibodies (above) were applied to a 2-ml column of normal human IgG coupled to CNBr-activated Sepharose (10 mg IgG/ml Sepharose). Both the effluent, containing anti-(mouse-derived)-IFX, and the eluate, eluted with citric acid and containing anti-(human-derived-IFX)-antibodies, were collected and analyzed. The presence of neutralizing anti-IFX antibodies in the effluent and eluted fractions was analyzed by the ELISA described above in combinations with ELISAs estimating their reactivities with normal human IgG and mouse IgG. The first two fractions of the effluent (F2 and F3) had minimal reactivity with human IgG but large inhibitory capacity; F3 was subsequently referred to as anti-(mouse-derived)-IFX antibodies. The purity was analyzed by SDS-PAGE and the concentration was estimated by the absorbance at 280 nm using the relationship $E^{1‰}_{1cm280nm}$=1.35.

Estimations of reactivity with human and mouse IgG. ELISA plates were coated with normal human IgG (0.2 μg/ml; Sigma) or mouse IgG (1 μg/ml; Sigma) and incubated with dilutions of the fractions obtained from the chromatographic depletion of anti-(human-derived)-IFX antibodies. Bound antibodies were detected by means of HRP-labeled swine anti-rabbit-IgG (2 μg/ml) and developed with OPD.

Results

Responses after IFX administration. Four rabbits were immunized with IFX, two intravenously (*i.v.*1 and *i.v.*2) and two subcutaneously (*s.c.*1 and *s.c.*2). Twelve days after the fourth and final IFX administration the rabbits were exsanguinated and the obtained plasma was used to develop assay technologies (described below). To test the reactivity, the plasma samples were analyzed for anti-IFX antibodies (Figure 3A) and for their potential to inhibit IFX-reactivity with TNFα (Figure 3B). We found that three out the four rabbits had mounted a robust antibody response towards IFX with development of neutralizing ADAs that target the antigen-binding site of IFX, inhibiting IFX's interaction with TNFα. Of the three ADAs-positive rabbits, the highest antibody titers were seen in rabbit *s.c.*1 and *s.c.*2, with *s.c.*1 showing the strongest development of neutralizing antibodies. One of the rabbits, *i.v.*1, possessed tolerance towards IFX and did not develop anti-IFX antibodies.

Fractionation of anti-IFX. As IFX is a chimeric mouse and human antibody, we expected that the anti-IFX reactivities, observed in three of the four rabbits at the time of exsanguination, would consist of a mix of anti-(mouse-derived)-IFX antibodies and anti-(human-derived)-IFX antibodies (see definition in Figure 1). To separate these two pools of antibodies, a two-step fractionation scheme was applied on the plasma obtained from rabbit *s.c.*1. The total pool of anti-IFX antibodies was purified by affinity chromatography using an IFX column (Figure 2) and the eluate was applied to a human IgG column (Figure 4).

Through the initial IFX-immunochromatography we found that >85% of the total reactivity was retained in the eluate (not shown) and that the eluate still contained a large proportion of neutralizing antibodies The purity of the eluates was estimated by SDS-PAGE and Coomassie staining to be >90% (Figure 2). A pool of fractions in this eluate (fractions 16-21; Figure 2) was used as a standard to estimate absolute concentrations of anti-IFX antibodies (see below).

In the second fractionation (on the human IgG column) we found that both the effluent and eluate contained neutralizing antibodies towards IFX. Taking the total IgG concentration into consideration, the largest specific activity of IFX neutralization was observed in the effluent (Figure 4A). The reactivity of effluent and eluate for mouse and human IgG was also analyzed by ELISA, respectively, and it was evident that the largest reactivity towards human IgG was localized in the eluate but that the column had a limited capacity to deplete anti-human-IgG antibodies (Figure 4B). Fraction numbers 2 and 3 of the effluent possessed highest specific activity with minimal reactivity with human IgG and were considered to be depleted of anti-(human-derived)-IFX antibodies. Fraction number 3 is from hereon referred to as anti-(mouse-derived)-IFX antibodies. Fraction number 17 is from hereon referred to as anti-(human-derived)-IFX antibodies. The purity of these fractions was found to be >90% and, for standardization purposes, the neutralization potential of the two pools was analyzed using inhibition ELISAs (Figure 5). We found parallelism between curves obtained for the pool of anti-(mouse-derived)-IFX antibodies and for that of unfractionated plasma and anti-IFX antibodies, obtained by the initial immunochromatography on an IFX column. The

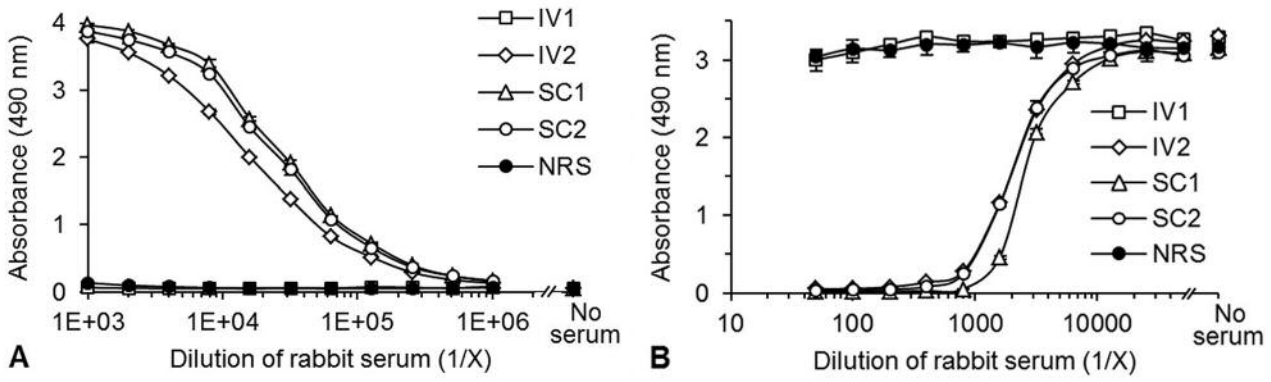

Figure 3. *Anti-infliximab (anti-IFX) antibody responses and their ability to inhibit IFX activity. A) Anti-IFX antibody responses by ELISA using IFX for coating and detection with HRP-conjugated anti-human IgG in the four rabbit administered IFX by i.v. (i.v.1 and i.v.2) or s.c. (s.c.1 and s.c.2) administrations, in comparison to a normal rabbit serum (NRS). B) Inhibition of IFX activity of the same rabbits by inhibition ELISA using IFX for coating and biotinylated-TNFα/streptavidin-HRP for detection. Data represent the average of triplicate measurements with error bars corresponding to maximum and minimum values. Note: i.v.2 and s.c.2 gave nearly identical inhibition values.*

parallelism demonstrates that the anti-(mouse-derived)-IFX antibodies and the pool of anti-IFX antibodies can be used as standards for correlation to absolute concentrations of IFX-neutralization antibodies and anti-IFX antibodies, respectively.

Assessment of functional IFX activity during IFX administration. By using an ELISA with immobilization of streptavidin/biotinylated TNFα and detection of bound IFX by HRP-conjugated anti-human-IgG, we measured concentrations of functional IFX in plasma samples obtained during the period of IFX administrations spanning 54 days (Figure 6). Two hours after the first *i.v.* IFX administration, we observed levels of 80-90 μg IFX /ml, corresponding to the levels in inflammatory bowel disease (IBD) patients receiving IFX treatments. Within 1-3 days, the concentration of functional IFX was reduced by 50% and, at day 7, 15-25% still remained in the circulation. After the third *i.v.* administration and development of IFX antibodies in rabbit *i.v.*2 (but not in *i.v.*1), we observed only a maximum of 40 μg/ml, 2 hours post administration, with a rapid reduction to 10 μg/ml, already at day 1 and undetectable levels at day 3.

IFX administrations by the *s.c.* route resulted in only minimal levels of functional IFX in the circulation that peaked at day 3, with 0.25-0.43 μg IFX/ml. The observed minimal of IFX is likely due to the adjuvant used for *s.c.* administrations. Subsequent *s.c.* administrations, after the development of anti-IFX antibodies, decreased levels of functional IFX to below 0.02 μg/ml. The method for assessment of functional IFX activity was directly applicable to human serum with minimal background signals and a detection limit of 1.5 μg IFX /ml (Figure 6C).

Assessment of anti-IFX antibodies during IFX administration. To correlate the disappearance rate of functional IFX in the circulation with the development of antibodies, we measured the concentrations of total anti-IFX and neutralizing anti-IFX antibodies using two different assays calibrated with purified preparations of anti-IFX antibodies and anti-(mouse-derived)-IFX antibodies, respectively (Figure 7). Development of anti-IFX antibodies was detectable at day 14 after only a single IFX administration; although levels were relative low, 24-60 μg/ml. At day 42, after two rounds of administrations, both rabbits receiving *s.c.* administrations had developed antibody levels between 900-1,800 μg/ml. Rabbit *i.v.*2, receiving *i.v.* administrations, was slower by comparison and mounted an antibody level of only 400 μg/ml at day 45, two days after the 3rd administration. It was evident that rabbit *s.c.*2 during administration program was superior to *s.c.*1 in terms of anti-IFX antibodies.

In terms of timing of the responses, the development of neutralizing anti-IFX antibodies was concomitant with the development of anti-IFX antibodies (above). At days 42-45 the levels of rabbits *s.c.*1 and *s.c.*2 were approximately equal at 700-800 μg/ml, whereas *i.v.*2 had an antibody level of 400 μg/ml. At day 54 the levels among the three responding rabbits were more or less equal at 1,100-1,400 μg/ml, regardless of administration route.

Discussion

By generating polyclonal antibodies in rabbits and by their further fractionation, we have made two relatively simple assays for the absolute measurements of functional concentrations of therapeutic antibodies in circulation and of the concentrations of neutralizing antibodies directed against

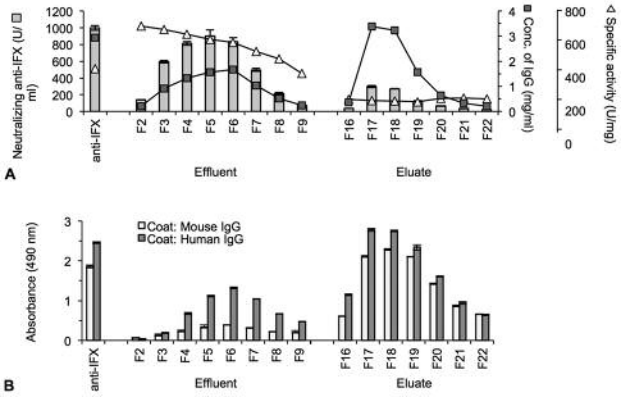

Figure 4. *Purification of anti-(human-derived)-infliximab (IFX) antibodies and anti-(mouse-derived-IFX) antibodies. A) Relative concentrations of neutralizing anti-IFX antibodies (grey bars, U/ml)) given by the reciprocal of the dilution giving 50% inhibition using an inhibition ELISA with IFX for coating and biotinylated-TNFα/streptavidin-HRP for detection. Total IgG concentration (filled squares, mg/ml) was measured by the absorbance at 280 nm and the specific activity (open triangles, U/mg) was calculated as: Relative concentration of neutralizing anti-IFX antibodies/total IgG. B) Anti-IFX reactivity with mouse and human IgG by ELISA using mouse and human IgG for coating and HRP-conjugated swine anti-rabbit IgG for detection. Data represent the average of triplicate measurements, with error bars corresponding to maximum and minimum values.*

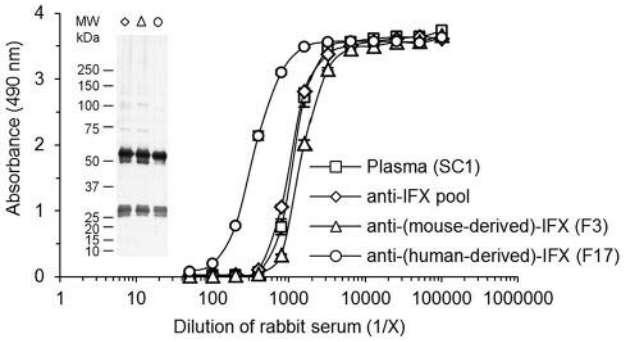

Figure 5. *Purity of antibody preparations and test of parallelism in infliximab (IFX)-activity inhibition ELISA. Test of parallelism for estimations of concentrations of neutralizing anti-IFX by inhibition ELISAs using IFX for coating and biotinylated-TNFα/streptavidin-HRP for detection. Purity of the fractions was assessed by SDS-PAGE and with silver staining. Data represent the average of triplicate measurements, with error bars corresponding to maximum and minimum values.*

the therapeutic antibody. Our administrations of the therapeutic antibody, IFX, in rabbits also illustrated how diverse tolerance and immune response may develop in patients receiving IFX treatments.

Three out of the four rabbits administered IFX reacted by producing ADAs. Judged from the immunoaffinity purification, the responses were in the form of IgG antibodies (Figures 2 and 4). The fourth rabbit presumably developed tolerance towards IFX, with an IFX half-life of 1-3 days, which was not influenced by repeated administrations of IFX; in contrast with the three other rabbits wherein, the half-life decreased as a function of the number of administrations and the development of antibodies (Figures 5 and 6). In comparison, the half-life of IFX in humans is 7-12 days, with the decreased half-life in rabbits probably reflecting the higher metabolic rate of smaller animals.

With the purpose of generating the necessary assays and standards for measurements of absolute concentrations of antibodies we fractionated rabbit anti-IFX plasma into pools of: anti-IFX, anti-(mouse-derived)-IFX and anti-(human-derived)-IFX antibodies (Figures 2, 3 and 4). The pool of anti-(mouse-derived)-IFX antibodies showed parallelisms with unfractionated plasma in the assay for measurement of neutralizing antibodies, allowing for its use as standard for absolute quantification of neutralizing antibodies (Figure 5). The low capacity of the column conjugated with human IgG could, unfortunately, only deplete anti-human IgG in the first two fractions of the effluent. It is likely that an increased column capacity would result in an increased sequestration of the anti-(human-derived)-IFX antibodies and, hence, in the generation of a larger amount of anti-(mouse-derived)-IFX antibodies to be used as standards.

The reactivity of the eluate and effluent showed also that anti-mouse IgG reactivity was located principally in the eluate and that the effluent had a relatively low reactivity with mouse IgG. The high reactivity of the eluate with mouse IgG is likely caused by cross-reacting rabbit antibodies that recognize human and mouse IgG equally well, due to shared epitopes, mainly located on the heavy chain of the two IgGs. The relatively low reactivity with mouse IgG in the effluent is likely due to lack of representation of mouse-derived IFX variable chain in the mouse IgG used for testing. It is unlikely that normal mouse IgG should include a large amount of IgG of the correct idiotypes, corresponding to the isotope of IFX. Based on the robust potential for neutralization in the effluent, there is no doubt that it contains a large amount of antibodies directed against the idiotope of IFX; however, these antibodies cannot be detected using normal mouse IgG for coating. It is also likely that the detected reactivity of the effluent with mouse IgG is due to antibodies raised against mouse specific non-idotypic epitopes in the mouse-derived light chain of IFX.

It is reasonable to assume that rabbit anti-idiotypic-IFX antibodies possess the highest potential to neutralize the activity of IFX. This may occur through direct interaction with the paratope of IFX. However, antibodies interacting

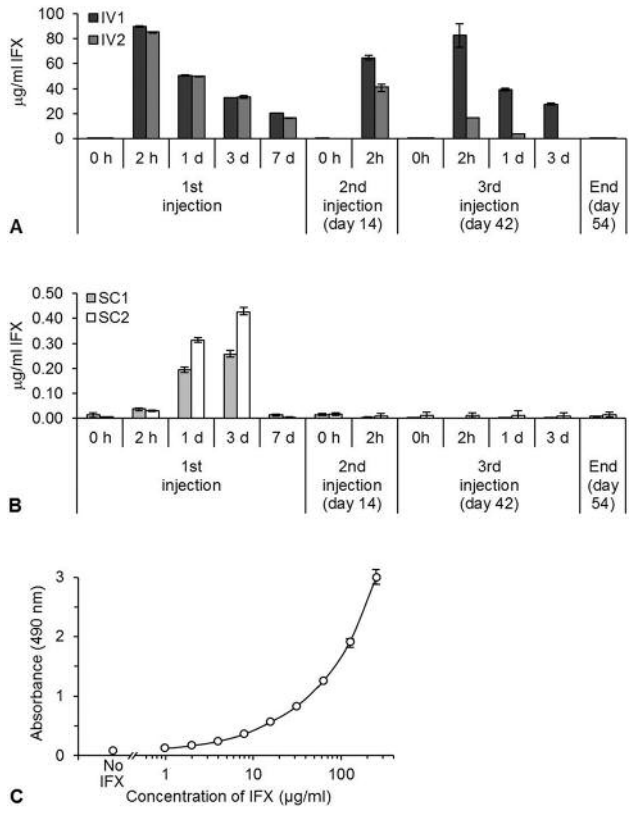

Figure 6. *Sequential measurement of functional infliximab (IFX) during IFX administrations. A) Functional plasma concentrations of IFX in rabbits receiving i.v. administrations as assessed by ELISA using immobilization of streptavidin/biotin-TNFα and detection with HRP-conjugated anti-human-IgG antibodies. B) Functional plasma concentrations of IFX in rabbits receiving s.c. administrations. C) Standard curve for assessment of functional IFX. Human plasma was diluted 1:1,000 and constituted with various concentrations of IFX. In absence of IFX, the background absorbance of the ELISA was measured to 0.073 (±0.001) and the lower detection limit was defined as the minimum. IFX concentration (data point) giving an absorbance of at least twice the background absorbance (2.0 µg IFX/ml, absorbance of 0.164 (±0.004)). The relative "high" lower detection limit of 2.0 µg IFX /ml plasma derives from the requirement of human plasma to be diluted 1:1,000, in order to obtain low background absorbance with optimal signal-to-noise ratio. Data represent the average of triplicate measurements, with error bars corresponding to maximum and minimum values.*

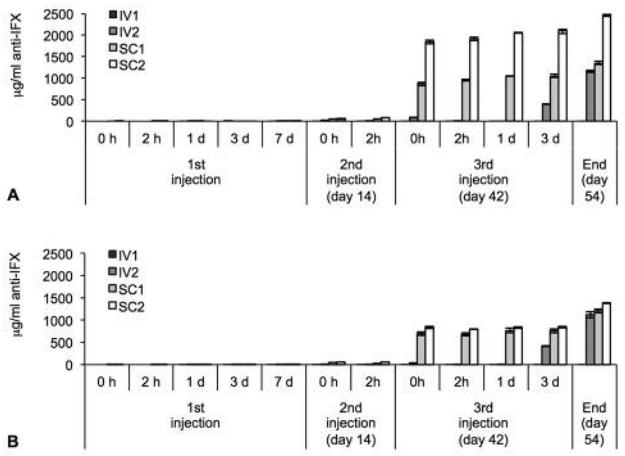

Figure 7. *Sequential concentrations of total and inhibiting anti-infliximab (anti-IFX) antibodies during IFX administrations. A) Concentrations of total anti-IFX antibodies as assessed by ELISA using IFX for coating and HRP-conjugated swine anti-rabbit-IgG antibodies for detection. For absolute quantification, a pool of immune affinity purified anti-IFX antibodies was used as standard (Figure 2). B) Concentrations of neutralizing anti-IFX antibodies by inhibition ELISA with IFX for coating and biotinylated-TNFα/streptavidin-HRP for detection. For absolute quantification a pool of immunoaffinity purified anti-IFX depleted of anti-human-IgG antibodies (anti-(murine-derived)-IFX antibodies) was used as standard (Figure 5).*

with either other idiotypic or allogenic epitopes located in proximity of the paratope of IFX, leading to steric hindrance of the interaction with TNFα have also great neutralizing potential. We observed that all three responding rabbits developed neutralizing, most likely anti-idiotypic, antibodies towards IFX. This observation shows that IFX is immunogenic and it is likely that the mouse-derived variable chains of IFX are just as immunogenic in humans as they are in rabbits.

The development of neutralizing antibodies in the three rabbits with ADAs correlated with increased disappearance rate of IFX. This may correspond to the situation in non-responders, among patients receiving IFX treatment. Thus, judged from the rabbit model, it should be possible to predict non-responders following the second IFX administration or, potentially, even earlier for primary non-responders.

The current assays should also be directly applicable in a human setting. The inhibition assay for estimating the concentration of neutralizing antibodies relies on inhibition of the binding of biotinylated-TNFα to immobilized IFX and should be directly applicable or, potentially, optimized using a pool of human anti-(mouse-derived)-IFX obtained from fractionation of non-responders, as a standard for the absolute quantification. The ELISA for estimating levels of functional IFX with a detection limit of 1.5 µg/ml should also be sufficient for analyzing patients' blood samples. Thus, our two assays represent valuable tools that can easily be adapted in clinical settings for personalizing and optimizing IFX treatment in patients.

Furthermore, as it only requires a pool of polyclonal antibodies and biotinylation of the biological target, in this case TNFα, the principles of the assays are easily adaptable to other therapeutic antibodies.

With the development of fully humanized therapeutic antibodies, one may question if patients in the future will develop immunogenic responses toward such therapeutic antibodies. However, treatments with fully humanized therapeutic antibodies, *e.g.* adalimumab, show that patients over time may develop ADAs and become non-responders of the drug (21, 22). The ADAs in such patients are likely to be directed against non-conserved residues (allogenic) in the constant parts of the light and heavy chains or even against the idiotopes of the therapeutic antibodies. One may even speculate that anti-idiotypic antibodies against exogenous antibodies (the administered therapeutic antibody) fail to be suppressed by "network" regulation, as they would by the B cells in the host. This could be due to the absence of idiotypes on the surface of B cells, in the form of membrane-localized immunoglobulins in a B cell receptor complex. Thus, testing immune responses of therapeutic antibodies seems relevant at present and also in the future.

To our knowledge, this is the first work to address absolute concentrations of functional IFX and of neutralizing antibodies. Other works have focused only on anti-IFX antibodies and/or the amount of IFX in circulation without, however, focusing on the functionality and without allowing for absolute concentrations of the two parameters (21-26). Implementation of the two assays for clinical settings is an ongoing process in our lab and will hopefully lead to better-personalized treatments in form of early identification of non-responders, optimization of dose regimens and termination of inadequate IFX therapy.

Conflicts of Interest

All Authors declare that they have neither financial nor commercial conflicts of interest in the study.

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