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Monitoring patients treated with anti-TNF-α biopharmaceuticals: assessing serum infliximab and anti-infliximab antibodies

M. Svenson[1,2], P. Geborek[3], T. Saxne[3] and K. Bendtzen[1,2]

Objectives. Infliximab is an anti-tumour necrosis factor-α (TNF-α) mouse–human IgG1/κ antibody used to treat patients with rheumatoid arthritis (RA) and other inflammatory diseases. Unfortunately, response failure and side-effects due to immunogenicity of the drug are not rare. In this study, we have compared different methods of assessing drug levels and anti-infliximab antibodies (Abs) and analysed the character of these Abs in sera of RA patients treated with infliximab for 1.5–18 months.

Methods. Functional serum infliximab levels and anti-infliximab Abs were measured by fluid-phase RIAs using ^{125}I-labelled ligands in combination with molecular size and affinity chromatography, and immune complex precipitation.

Results. Anti-infliximab Abs were predominantly IgG, 36% being IgG4, and half the immune complexes were λ-light-chain-positive. Ab titres were associated with inhibition of TNF binding to the drug, and low trough levels of infliximab were most frequent in anti-infliximab Ab-positive sera. Cross-binding to two other anti-TNF drugs was not observed. Detection of anti-infliximab Abs by solid-phase RIA using cross-binding of plastic-fixed and soluble infliximab exhibited low sensitivity and the data were inconsistent with results obtained from binding of the Abs to soluble infliximab.

Conclusions. Specific and neutralizing anti-infliximab antibodies develop in RA patients treated with infliximab, and that low trough levels of functional infliximab are associated with the presence of such antibodies. The most sensitive antibody assay involved binding to soluble and intact infliximab. Assessments of bioavailability and immunogenicity of anti-TNF biologicals may be used to optimize dose regimens and prevent prolonged use of inadequate therapy.

KEY WORDS: Rheumatoid arthritis, Tumour necrosis factor-α, Antibodies, Radioimmunoassay, Anti-allotypic antibodies.

Introduction

Anti-tumour necrosis factor (anti-TNF) therapy has become an important alternative in the management of chronic inflammatory diseases, including rheumatoid arthritis (RA). The first anti-TNF drug approved for clinical use was infliximab, a mouse-human IgG1:κ-chimeric anti-TNF-α monoclonal antibody (mAb). It binds to TNF-α and prevents the cytokine from triggering the cellular TNF receptor complex [1]. Unfortunately, 30–40% of the patients are primary non-responders [2], and dose- or dose frequency adjustments due to insufficient responses are frequently necessary [2–6].

Lack of treatment response has been attributed to immunogenicity. Hence, many patients with secondary response failure to one anti-TNF drug have benefited from a switch to other anti-TNF drugs, and the development of antibodies (Abs) is associated with secondary response failure and low circulating drug levels [5–13].

Several different methods have been used to assess circulating levels of infliximab as well as anti-infliximab Abs. Most of these are based on ELISA using specific Abs both for capture and detection, but also plastic-fixed ligands for capture and competitive-type ELISAs [4, 7, 10, 14]. Anti-infliximab Abs (IgG isotype) have also been detected by binding to ^{125}I-infliximab Fab2, and we have developed a fluid-phase RIA for anti-infliximab Abs (all isotypes) using ^{125}I-infliximab followed by separation of anti-infliximab Abs by anti-human λ light-chain mAb [5, 6].

There is, at present, little information on the clinical potential of these assays. We have, therefore, compared fluid-phase and solid-phase assays for circulating infliximab and anti-infliximab Abs. We have also monitored binding characteristics of anti-infliximab Abs generated in RA patients.

Methods

Patients and sera

All patients were cared for according to the protocol of the South Swedish Arthritis Treatment Group [15]. The follow-up of biological treatments in Sweden is part of a nationwide study imposed by the Swedish authorities including the Medical Products Agency. All patients were informed orally and in writing and gave their consent to participate. The infliximab dosage was in all cases 3 mg/kg but had to be increased in several patients because of insufficient efficacy. Sera were obtained from one group of 106 randomly selected RA patients as detailed [6]. These sera were collected after 1.5 months ($n=74$), 3 months ($n=75$) and 6 months of treatment ($n=63$). Another group of 43 RA patients had sera collected at various times from before (day 0) to 18 months after start of therapy; 25 of these sera were also included in the randomized sample. Sera were in all cases collected immediately prior to the next infusion of infliximab. Sera from five RF-positive and five RF-negative patients were also colleced, as was a pool of sera from 20 normal healthy donors (Blood Bank, Rigshospitalet, Copenhagen, Denmark).

Immunoreagents and infliximab

Murine mAbs against human Ig-allotype G1m(a) and G1m(x) were kindly provided by Red Cross Blood Transfusion Service, Amsterdam, The Netherlands [16]. Anti-IgG4 mAb G4.T9 was from ALK-Abello (Hørsholm, Denmark). All other reagents were collected as described [6].

Affinity and size chromatography

Abs were coupled to activated Mini-Leak Low agarose beads (Kem-En-Tec, Copenhagen, Denmark) and packed into 0.5 ml PD10 mini-columns (Amersham Bioscience/GE Healthcare, Hillerød, Denmark). Anti-IgG4 columns were as described [17].

[1]Institute for Inflammation Research, Rigshospitalet University Hospital, Copenhagen, [2]BioMonitor ApS, Copenhagen, Denmark and [3]Department of Rheumatology, Lund University Hospital, Lund, Sweden.

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Correspondence to: K. Bendtzen, Institute for Inflammation Research IIR7521, Rigshospitalet National University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. E-mail: kben@mail.dk

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For analytical purposes, 100 μl of sample was applied on the column followed by 2×100 μl washing buffer: phosphate-buffered saline (PBS), 0.025% gelatin, 0.1% Triton X-100. Then, 2.7 ml buffer (fraction 1) plus 3 ml buffer (fraction 2) was applied. Fraction 3 was eluted with 3 ml 0.1 M glycine–HCl, pH 2.4, 0.5% human serum albumin (HSA). The column was finally washed with 3 ml buffer (fraction 4). Percentage bound (B)/total (T) = 100 × (c.p.m. of fraction 3 + 4)/(c.p.m. of fraction 1 + 2 + 3 + 4). For preparative purposes, 200 μl sample was applied on the column followed by 2×100 μl assay buffer: PBS, 0.5% HSA, 5 mM EDTA. Then, 5 ml assay buffer was added, and the eluate was collected in 1 ml fractions. Bound material was collected in tubes with 20 μl 1 M Tris, pH 8, by successive addition of 12×0.5 ml 0.1 M glycine, pH 2.4. Size separation for purification of radiolabelled intact infliximab or Fab fragments was made on a PD-10-Sephacryl S300HR column (Pharmacia, Uppsala, Sweden), and size separation of free and bound infliximab was carried out on a 0.9×32 cm Sephacryl S300HR column using PBS buffer, 0.5% HSA, 0.01% NaN_3. The large column was calibrated with molecular weight (MW) markers (Amersham Bioscience); with catalase (MW 232 kDa) as marker, 10% was eluted in fractions 42 and higher. Percentage bound (B) ^{125}I-infliximab was $100 - z\ (1 + b/a)$, where z is the sum of % c.p.m. in fractions 42–60, a is the sum of % c.p.m. in fractions 42–60 and b is the sum of % c.p.m. in fractions 36–41. The sum of activities in fractions 20–60 was set to 100%. The value of b/a was 0.95 ± 0.1 (mean ± S.D., $n = 4$). ^{125}I-infliximab, 0.2 ml, containing 40 000 c.p.m. was used in each experiment.

RIA for TNF-α binding to serum IgG

Serum duplicates were diluted to 1% in assay buffer, or further in the presence of 1% normal serum. ^{125}I-TNF-α (NEX257; Perkin Elmer Life Science, Boston, MA, USA), 5000–6000 c.p.m., was added to 100 μl. After 2 h at 4°C, rabbit anti-human Fcγ was added in an amount capable of precipitating >95% of available IgG. After another 2 h, 2.5 ml ice-cold assay buffer was added, and bound and free ^{125}I-TNF-α were separated by centrifugation at 4000 g for 10 min at 4°C. The pellet activity was measured with an error <2% using an automatic gamma counter. When spiking the sample, infliximab was pre-incubated with 1% serum for 18 h at 4°C, and binding of ^{125}I-TNF-α was tested as described earlier in the article. The assay was performed with an inter- and intra-assay error of 20 and 10%, respectively.

RIA for anti-infliximab Abs

Infliximab was labelled with ^{125}I using chloramin-T [18], purified by molecular size chromatography, and tested for binding to different Ig immunoreagents (Table 1). ^{125}I-infliximab, 3000–4000 c.p.m./100 μl and specific activity $1–2 \times 10^5$ c.p.m./ng, was co-incubated for 18 h at 4°C with 1% or lower levels of serum. The anti-infliximab activity was then assayed by binding of ^{125}I-infliximab to an affinity matrix containing anti-human λ-light-chain Ab capable of binding >80% of λ-light-chain immunoglobulins in 10% serum. The mean background binding was $2 \pm 0.3\%$ ($n = 20$), and two times the background value was used to discriminate between positive and negative samples.

TABLE 1. Binding of ^{125}I-infliximab and ^{125}I-infliximab Fab fragments to Ig-binding reagents

			Abs to:		
	Protein A	Protein G	Fcγ	λ-light-chains	κ-light-chains
Infliximab	0.94 ± 0.05	0.96 ± 0.04	0.95 ± 0.04	0.03 ± 0.01	0.93 ± 0.04
Infliximab-Fab	0.03 ± 0.01	0.92 ± 0.03	0.04 ± 0.01	0.03 ± 0.01	0.92 ± 0.03

Reactivity was tested as B/T radioligand (mean ± S.D.; $n = 4$) using saturation levels of Ig-binding reagents.

Assays for infliximab Fab and Fc fragments, and Ig allotypes

Infliximab, 10 mg in 1 ml PBS, 2 mM EDTA, was incubated for 18 h at 37°C with 20 mg activated papain-agarose (Sigma, St Louis, MO, USA). The supernatant, termed papain-infliximab, was collected after centrifugation and stored at 4°C until use. ^{125}I-infliximab, 10^7 c.p.m., was similarly digested using 4 mg activated papain-agarose and 6 h of incubation.

Recovered activities of infliximab Fab and Fc fragments were assessed by competition using specific antibodies fixed to ELISA Maxisorp plates (Nunc, Roskilde, Denmark). The concentrations of rabbit anti-γ-chain Ab and anti-κ-chain Ab were 0.6 μg/ml and 2.5 μg/ml, respectively, and the anti-allotype mAbs MG102 and MG105 were fixed at 0.3 and 2 μg/ml, respectively. The assays were performed by adding 75 μl/well of serial dilutions of the samples in assay buffer followed by addition of 50 μl/well of ^{125}I-infliximab. After incubation at 4°C for 18 h, the wells were washed, and bound c.p.m. was solubilized by 2×200 μl/well of 2.5 N NaOH, 0.5% HSA and counted. The sensitivities of these four assays were from 0.005 to 0.01 μg infliximab/ml.

Purification of infliximab Fab and Fc fragments

Papain-infliximab was separated on protein A Sepharose into two pools containing either Fab or Fc. Each pool was then subjected to affinity chromatography using anti-human κ-light-chain Ab. The Fc activity was collected in the fractions of non-bound material, and the Fab activity in the acid-eluted fractions.

^{125}I-Fab infliximab was purified by first applying 300 μl of the papain-^{125}I-infliximab; for molecular size separation on a PD-10 column, see above. Fractions containing molecules from 70 to 30 kDa were pooled and recirculated on a protein A Sepharose column with Fab activity recovered in the non-bound fraction.

RIA for IgG anti-infliximab Fab

Duplicates of patient sera, tested at 1% or further diluted in 1% normal serum, were incubated with 3000–4000 c.p.m. ^{125}I-infliximab Fab per 100 μl. After 18 h at 4°C, rabbit anti-human γ Ab was added. After 2 h, 2.5 ml ice-cold assay buffer was added, and free and IgG-bound ^{125}I-infliximab Fab were separated by centrifugation at 4000 g for 10 min at 4°C. Background activity was $4 \pm 0.5\%$ ($n = 12$) when detected with 1% serum and excess infliximab.

Solid-phase RIA for anti-infliximab Abs

Infliximab, 8 μg/ml, was fixed to ELISA Maxisorp plates followed by blockade of non-occupied sites with HSA. Sera were added in duplicate, using 10% or lower levels and 100 μl/well. After overnight incubation at 4°C, the wells were washed with ice-cold assay buffer, and 100 μl of 6000 c.p.m. of ^{125}I-infliximab was added per well. After 3 h at 4°C, the wells were washed and the bound c.p.m. was determined. Background binding was $0.5\% \pm 0.04$ ($n = 20$) when detected in the absence of serum.

Results

Binding of infliximab to Ig-binding reagents

While reacting with all other reagents tested, infliximab failed to bind to anti-human λ-light-chain Abs (Table 1). The Fab fragments of infliximab also bound to protein G, but not to protein A. As expected, there was no infliximab-Fab binding to anti-Fcγ mAbs.

Serum infliximab and anti-infliximab Ab

^{125}I-TNF-α failed to bind to sera before infliximab therapy ($n = 43$). During therapy, however, patient sera exhibited ^{125}I-TNF-α binding, and the tracer could therefore be used to quantify functional levels of infliximab [6]. Anti-infliximab Abs were assessed semi-quantitatively as the amount of ^{125}I-infliximab that bound to serum and, subsequently, to an anti-λ Ab affinity matrix [6].

As shown in Fig. 1, trough serum infliximab levels were distributed in a scattered fashion with the lowest levels in Ab-positive sera. While the median levels of infliximab in the Ab-negative sera exceeded 2 μg/ml at all time points, median levels were always below detection in the Ab-positive sera.

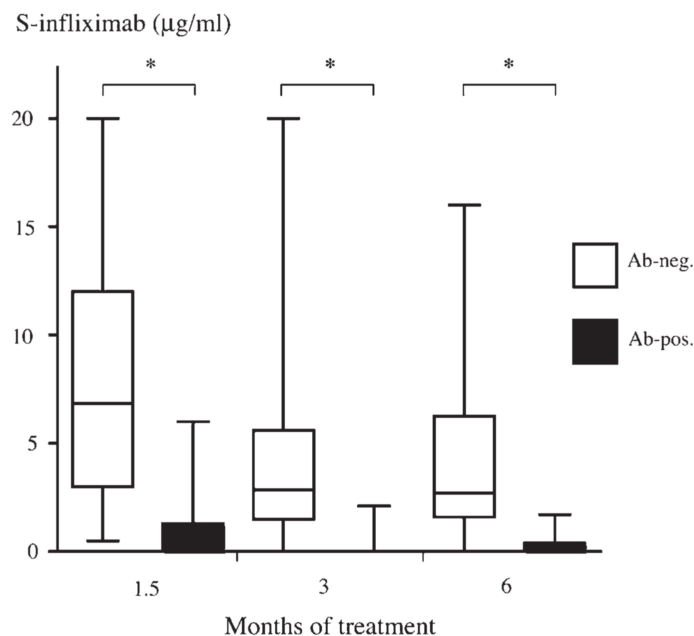

Fig. 1. Trough levels of functional infliximab in sera positive or negative for anti-infliximab Abs. Sera were collected from a cohort of 106 randomly selected RA patients at the indicated time points after start of infliximab therapy; 74 sera were available at 1.5 months, 75 at 3 months and 63 at 6 months. The levels of functional infliximab were calculated from the detected binding of ^{125}I-TNF-α. Anti-infliximab Abs were measured by fluid phase RIA detecting ^{125}I-infliximab in complex with λ-light-chain-positive Abs. The limit for Ab-positivity was set to 2× background. Results are shown as medians (ranges/quartiles). $^{*}P < 0.0001$ (Mann–Whitney rank sum test).

Comparing RIAs for anti-infliximab and anti-infliximab-Fab Abs

The above assay for anti-infliximab Abs took advantage of the absence in infliximab of λ-light-chains. However, the Ab response to infliximab is expected to include immunoglobulins containing κ-light-chains as well, and it is therefore possible that only a fraction of bound ^{125}I-infliximab may be found in complex with anti-λ Abs. We, therefore, compared Ab binding to ^{125}I-infliximab using both anti-λ affinity chromatography and molecular size chromatography. We first ascertained a similar stability of ^{125}I-infliximab complexes in the two assays: after 3 h in excess of unlabelled infliximab, complex dissociation was 35% (21–45) and 32% (19–45), respectively, $n = 6$. As shown in Fig. 2A, none of the Ab-positive sera tested contained immune complexes above 670 kDa, the peaks of eluted complexes appearing between 440 and 670 kDa. The anti-infliximab Abs were dominated by the IgG isotype, because absorption of six randomly chosen sera to an anti-human IgG Ab matrix abolished binding of ^{125}I-infliximab detected by molecular size chromatography. There was a highly significant linear correlation between the two assays, and there was a significant correlation between total and IgG4-bound infliximab with a relative contribution of IgG4-positive complexes of 36% (8–89%); median (range), $n = 14$ (Fig. 2B).

We next tested the binding of ^{125}I-infliximab-Fab to serum IgG. To compare with the anti-λ RIA, we selected sera that bound ^{125}I-infliximab at some time points up to 12 months of therapy. Of a total of 147 sera from 27 patients tested, 68 sera were judged positive for anti-infliximab Abs, and 48 of these (71%) were also positive for anti-infliximab-Fab Abs (Fig. 3A and B). The highest binding values (B/T) were detected by the anti-infliximab-Fab RIA with values above 90% compared with maximum values of almost 50% in the anti-λ RIA. Plotting B/T from the two assays revealed a scattered distribution, but with a highly significant positive correlation (Fig. 3C). When testing sera with B/T >25% in the anti-infliximab assay, B/T for anti-infliximab-Fab Abs varied from less than 10% to 90%. Furthermore, 50% were judged positive at 2–3 months, 1–2 months before they were detectable by the anti-infliximab-Fab assay.

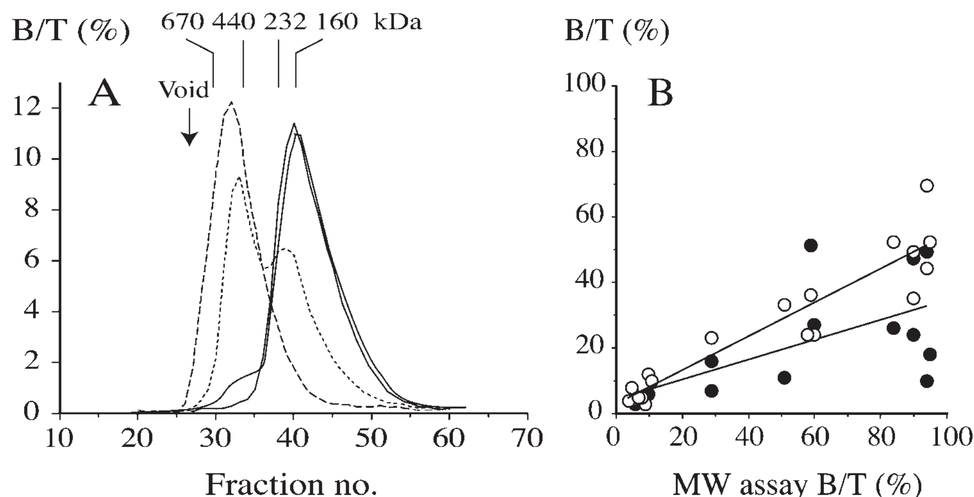

Fig. 2. Serum binding of ^{125}I-infliximab. Size distribution and correlation between two different RIAs. (**A**) Elution profiles of ^{125}I-infliximab after pre-incubation in sera from patients treated with infliximab for 3 or 6 months and subsequently analysed by Sephacryl S300HR chromatography. The figure shows four representative profiles of 14 randomly selected Ab-positive sera. (**B**) ^{125}I-infliximab bound to λ-light-chains (open symbols) and to IgG4 (closed symbols) plotted against ^{125}I-infliximab assessed by MW separation (see M + M). Linear correlation for open symbols: Spearman's $r = 0.92$, $P < 0.0001$, $n = 19$. Slope = 0.51. Linear correlation for closed symbols: Spearman's $r = 0.64$, $P = 0.01$, $n = 14$. Slope = 0.30.

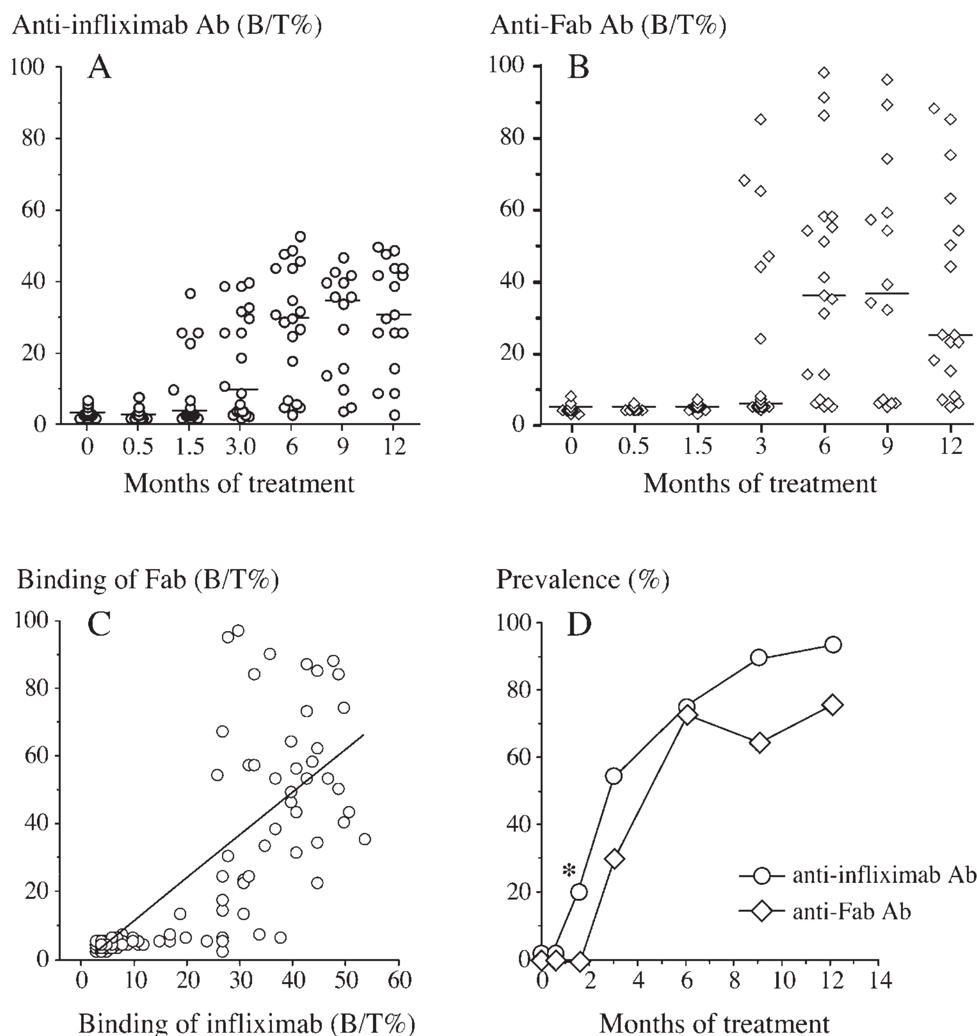

Fig. 3. Anti-infliximab Ab binding and prevalence tested by two different RIAs. (**A**) RIA using ^{125}I-infliximab binding to anti-λ light-chains. Sera were selected from 27 RA patients treated with infliximab for up to 12 months and detected Ab-positive at least at one time point during therapy. A minimum of 14 sera were tested at each time point. (**B**) RIA using ^{125}I-infliximab-Fab binding to IgG. Same sera as in A. (**C**) Correlation between the RIAs. Linear correlation: Spearman's $r = 0.75$, $P < 0.0001$, $n = 147$. (**D**) Prevalence of anti-infliximab-positive patients; Ab-positivity was defined as in Fig. 1. $^{*}P = 0.05$, Fisher's exact test.

Cross-binding of infliximab-Fc and -Fab fragments to anti-infliximab Abs and relative sensitivity of the assays for Abs to infliximab-Fab and native infliximab

Different results obtained by RIAs for Abs to infliximab-Fab fragments and to native infliximab can be attributed to the different sensitivity/efficacy by which they detect the immune complexes but also to partial cross-binding of infliximab-Fab and native infliximab to the Abs. To test the latter, we used purified infliximab Fab and Fc fragments. As shown in Table 2, papain treatment of infliximab resulted in recoveries of 20–80% depending on the Ab reactivity tested. However, with the use of papain-infliximab as reference, the purified Fc and Fab showed full recovery with <1% cross-reactivity. Papain-infliximab was therefore selected as reference in an analysis of Fab and Fc binding to anti-infliximab Abs. Using an equivalent activity of Fab and Fc fragments, and of papain-infliximab, competition experiments showed significantly lower cross-binding of the Fc part. Furthermore, the apparent cross-binding of the Fab parts occurred to >90% of the anti-infliximab Abs compared with <20% to the Fc part (data not shown). Complete cross-binding between infliximab and Fab was also observed in the anti-infliximab-Fab IgG assay because addition of 20 μg/ml of infliximab reduced the binding of

Table 2. Recovered activities following papain treatment of infliximab and of purified Fc and Fab fragments of the drug detected by different Abs

Infliximab components	Percentage recovered activity using Ab to: Ig Fcγ	κ-light-chain	Infliximab	Allotype G1m (a)	Allotype G1m (x)
Papain-infliximab	80	20	20	60	80
Fc fragments	80	<0.5	<0.5	60	70
Fab fragments	0.4	20	20	<0.5	<0.5

The activities were calculated from the use of RIA with infliximab as reference. All preparations with recovered activity of at least 20% were confirmed to completely block the binding of ^{125}I-infliximab in the assay. Results are mean percent recoveries of duplicate determinations of serial dilutions of the samples. For the explanation of the different Abs, see 'Methods' section.

^{125}I-infliximab-Fab to the background level: B/T without added drug: 0.53 (0.31–0.86) and B/T with added infliximab: 0.02 (0.015–0.03), $n = 10$. These results suggest that the same population of IgG binds to infliximab and Fab and that the sera tested contained few if any specific IgG molecules not sharing this binding.

To evaluate the relative contribution to Ab binding by the Fab and Fc fragments, variable concentrations of infliximab, Fab and Fc were tested for competition in the anti-infliximab Ab assay,

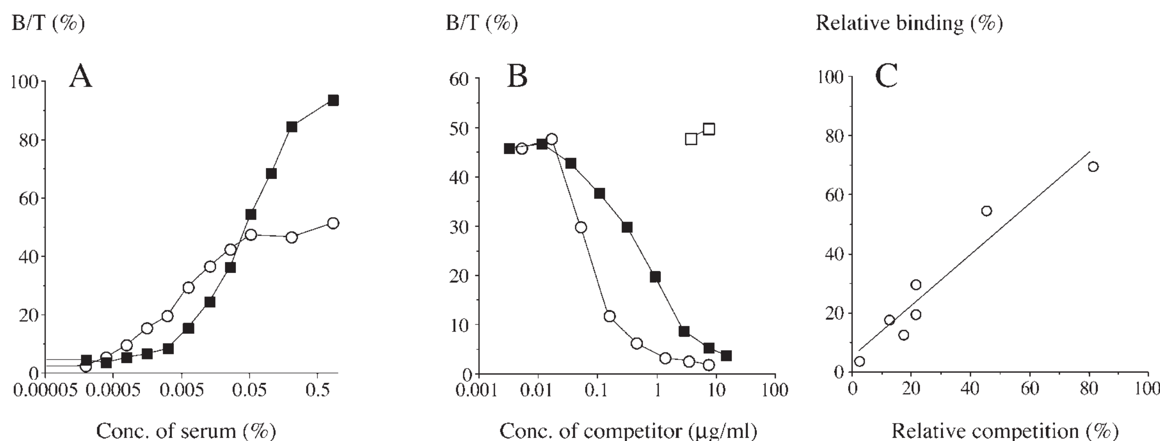

FIG. 4. ^{125}I-infliximab and ^{125}I-infliximab-Fab binding to anti-infliximab Ab-positive RA serum. (**A**) Binding of ^{125}I-infliximab to λ-light chains (open circles) and ^{125}I-infliximab-Fab to IgG (closed squares). Tests were carried out using serum from one anti-infliximab Ab-positive patient with $B/T = 52\%$ at 1% serum in the anti-λ assay. (**B**) Competition with ^{125}I-infliximab binding afforded by unlabelled infliximab Fc (open squares), Fab fragments (closed squares) or 'cold' infliximab (open circles). Same serum as in (A). (**C**) Correlation between relative binding and relative competition of infliximab-Fab and infliximab. The relative values were calculated from experiments similar to those shown in (A) and (B) using sera with B/T values of 23–53% at 1% serum in the anti-λ assay and variable levels of anti-infliximab Ab; see text for details. Slope: 0.86. Linear correlation: Spearman's $r = 0.96$, $P = 0.003$, $n = 7$.

TABLE 3. Prevalence of anti-infliximab Ab-positive sera detected by two assays

Type of assay	Positive sera (of 171 tested) (%)
Fluid-phase RIA (anti-λ Ab matrix)[a]	31 (18%)
Solid-phase RIA[b]	20 (12%)
Both assays	12 (7%)

Sera were judged positive when showing B/T 2× the background.
Sera collected from 43 patients treated up to 18 months with infliximab were tested as follows: [a]At 1% in assay detecting ^{125}I-infliximab binding to serum by absorption to an anti-λ light-chain Ab matrix. [b]At 10% by cross-binding of ^{125}I-infliximab to solid-phase-fixed infliximab.

together with tests of the binding activity of the same serum at serial dilutions. As shown in Fig. 4A and B, serum IgG bound primarily to Fab. In agreement with this, tests of sera of another five individuals showed that Fc expressed <1% of the Fab binding (data not shown).

In the competition assay, the relative binding strength of infliximab and infliximab-Fab was calculated as the concentration ratio at 50% inhibition, but because of differences in maximal binding in the direct binding assays, the relative binding strength was calculated as the mean of the ratios of serum levels giving 2, 4 and 8 times the background binding in the anti-infliximab and Fab assays, respectively.

As shown in Fig. 4, both assays exhibited the lowest activity with infliximab-Fab with activities ranging from a few percent to near equal activity of that of infliximab when looking at the binding to individual sera.

These results support the notion that the B-cell epitopes of infliximab are only or mainly located at the Fab-part of the drug and that there are substantial individual differences in the ratio between binding of intact infliximab and infliximab-Fab.

Solid-phase RIA of anti-infliximab Abs detected by cross-binding of soluble to fixed infliximab

Infliximab fixed to ELISA plates was used for absorption of anti-infliximab Abs, and multivalent Ab activity was then detected by binding to ^{125}I-infliximab. As shown in Table 3, fewer sera were detected positive using the cross-binding assay even at 10 times higher concentration than those tested by fluid-phase RIA. Moreover, 8/20 (40%) of sera found positive in solid-phase RIA were negative in the other anti-infliximab assay. However, the highest binding activities in the solid-phase RIA were found for sera positive in both assays: $B/T = 23 \pm 30\%$ ($n = 12$) when positive in both assays *vs* $2.7 \pm 1.5\%$ ($n = 8$) if positive only in solid-phase RIA.

Assay sensitivities and interference by RF on solid-phase RIA for anti-infliximab Ab

When testing two Ab-positive sera, Ab detection by solid-phase RIA was 700–3000 times lower than that of the fluid-phase RIA using anti-λ Ab, and the RIA for infliximab-Fab was 5–10 times less sensitive than the fluid-phase RIA.

While RF-positive sera failed to interfere with the fluid-phase RIA, they reacted in the solid-phase RIA. Thus, the median (ranges) B/T of 10% sera from five RF-negative RA patients (RF IgM <14 kIU/l) not treated with infliximab, and therefore anti-infliximab Ab-negative, was 0.33% (0.27–0.38), whereas B/T of 5 RF-positive sera [RF IgM = 390 (330–570) kIU/l] was 1.4% (0.71–4.8); $P < 0.01$ (Mann–Whitney test).

Specificity of therapy-induced anti-infliximab Abs

Two other anti-TNF human IgG1 constructs are presently approved for clinical use, etanercept and adalimumab [19]. We, therefore, tested whether binding of ^{125}I-infliximab to serum λ-light-chain-positive immune complexes was affected by these two anti-TNF constructs. While infliximab, 0.02 μg/ml and above, suppressed ^{125}I-infliximab binding, none of the 15 randomly chosen anti-infliximab-positive sera showed detectable cross-binding to 2 and 20 μg/ml of etanercept and adalimumab, respectively (data not shown).

Neutralization of TNF-α binding to infliximab by anti-infliximab Ab

As shown in Fig. 5A, adding 10 ng/ml of infliximab to a 1% normal serum pool allowed subsequent binding of nearly 40% of added ^{125}I-TNF-α. We compared this with 26 sera from RA patients treated for 6 months containing variable levels of anti-infliximab Abs (and <0.3 μg/ml infliximab). When 1% of the sera were spiked with 10, 100 and 1000 ng/ml of infliximab, more than 50% suppression was obtained in 11, 5 and 2 sera, respectively, indicating a competitive effect. As shown in Fig. 5B, there was a positive correlation between blockade of TNF binding to added infliximab (10 ng/ml) and the titre of the anti-infliximab Abs detected by use of anti-λ RIA. Setting the detection limit to 20% in the blocking assay, 18/26 (69%) sera testing positive by anti-infliximab fluid-phase RIA were also judged positive in

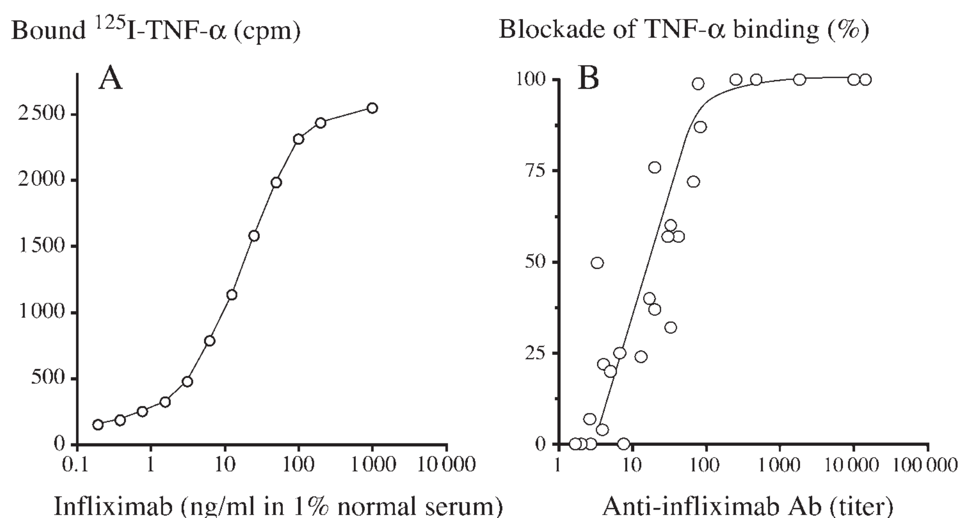

FIG. 5. Anti-infliximab Abs block TNF-α binding to infliximab. (**A**) Reference curve of ^{125}I-TNF-α binding to variable levels of infliximab added to 1% normal serum (anti-infliximab Ab-negative; pooled from 20 healthy individuals). Representative of four experiments. (**B**) Blockade of ^{125}I-TNF-α binding to infliximab by sera from 26 RA patients with variable levels of anti-infliximab Ab. Testings were carried out using 1% serum spiked with 10 ng/ml of infliximab. Titres were defined as the dilution of a 1% serum sample yielding two times the background binding of ^{125}I-infliximab to λ-light-chains.

blocking TNF binding to infliximab (Fig. 5B). Ten sera, double negative at 3 or 6 months of treatment, all showed full recovery when spiked with 10 ng/ml of infliximab. Furthermore, size separation of 1% serum confirmed the negative anti-infliximab data. However, at 5%, 7/10 (70%) were positive in both assays with a maximum of four times the background binding.

These results suggest that anti-infliximab-negative/low positive sera have little or no potential to block TNF binding to infliximab.

Discussion

The two light-chains of a natural mAb are of either the κ or λ isotype, and in normal human sera, the $\kappa:\lambda$ ratio is 2 : 1 [20]. There are no reports on deviations from this ratio in sera from RA patients. However, the ratio may differ to some degree with regard to specific Ab responses depending on the antigen and the avidity of the Abs generated [20, 21].

As infliximab is a human IgG1 construct reacting with a number of anti-human IgG reagents, but not with anti-human λ Abs, we assessed the development of anti-infliximab Abs during infliximab treatment by binding of ^{125}I-infliximab to an anti-λ affinity matrix. This should reveal immune complexes consisting of infliximab and λ Abs of all isotypes. Furthermore, we compared this with a more cumbersome assay using molecular size separation of unbound and Ig-bound infliximab with and without depletion of serum IgG. We found that the single-most important binding factor in serum of infliximab-treated RA patients is IgG and that about 51% of bound infliximab was in complex with IgG : λ Abs in these sera.

The anti-λ RIA was comparable in sensitivity to the molecular size separation RIA, and the stability of the detected complexes was similar in the two tests, suggesting that equal binding avidity is expressed by κ- and λ-positive anti-infliximab IgG Abs, and in line with the observation in mice that the light-chain isotype has a low impact on IgG-binding avidity [22].

The immune complexes generally consisted of maximum three anti-infliximab IgG antibodies, as their sizes were from 600 kDa and below. This may be explained by the observation that a significant portion of the induced anti-infliximab Abs are IgG4, a subclass known to form small complexes with low complement activating capacity and with lower avidity to FcγR than the other IgG classes [23]. Assuming that anti-λ Abs constituted 1/3 of Abs generated against infliximab (see above), the prevalence would be 0.33–0.71 for at least one λ-light-chain-positive IgG molecule in complex with infliximab when bound to one to three IgG Abs. Thus, the efficacy of the anti-λ RIA supports the view that therapy-induced anti-infliximab IgG Abs express κ- and λ light-chains at the normal ratio of 2 : 1 and that binding avidities are largely independent of the light-chain isotype.

We found the B-cell epitopes of infliximab to be mainly or solely located at the Fab parts of the drug. However, the relative IgG-binding avidity of the free Fab part compared with the intact molecule varied considerably among individuals. Thus, some individuals seem to develop Abs mainly directed to the epitopes influenced by papain treatment. Importantly, sera from 15 individuals with therapy-induced anti-infliximab Abs did not show detectable cross-binding to two other approved anti-TNF IgG1 constructs, etanercept and adalimumab. This shows that Abs generated in infliximab-treated patients are generally specific for this anti-TNF construct. This would explain why a shift to other anti-TNF drugs is effective in some patients with therapeutic failure or intolerance during infliximab therapy [7, 8, 11].

That Abs to infliximab are exclusively of the IgG isotype and primarily directed against the Fab parts of the drug is of relevance when comparing our results with a recent study where ^{125}I-labelled, pepsin-treated infliximab-Fab2 and protein A were used to monitor therapy-induced IgG anti-infliximab Abs in RA patients [5]. Even though this study would not have detected Abs directed against the Fc part of the drug, anti-infliximab Abs were detected in nearly half the patients after 1 yr of treatment, a result similar to that obtained in the present study assessing Abs to the whole molecule.

Spiking experiments revealed a positive correlation between reduced recoveries and the levels of anti-infliximab Abs, and sera tested negative for both functional infliximab and anti-infliximab Abs expressed full functional recovery of added infliximab. Thus, the assay procedures used in this study show no interference with TNF binding to infliximab by serum factors besides the Abs induced during treatment.

In agreement with other studies [6, 13], the anti-infliximab Ab-positive sera contained particularly low levels of infliximab. A possible explanation could be an increased clearing of the drug due to immune complex formation. Even non-neutralizing Abs might contribute to this effect.

When monitoring anti-infliximab Ab in RA patients, we favour the use of fluid-phase RIA rather than solid-phase cross-binding tests, whether RIA or ELISA types. There are several reasons for this:

- The fluid-phase RIA is more sensitive.
- The fluid-phase RIA is unaffected by RF: RF-positive pre-treatment sera were found to bind to infliximab, but only when assayed by the solid-phase cross-binding RIA. RA patients frequently express Abs against IgG allotypes, most commonly against G1m and often in association with RF activity [16, 24, 25]. Interestingly, we found that infliximab itself expressed the G1m(a) and G1m(x) allotype markers.
- The fluid-phase RIA also detects Abs that are functionally monovalent: functional multivalency is mandatory for a positive reaction in the solid-phase cross-binding RIA. This test would therefore not detect functionally monovalent Abs such as IgG4 [26], which might nevertheless neutralize TNF binding, increase drug clearance and/or prevent it from reaching the inflamed tissues. IgG4 constituted from 8% to 89% of the anti-infliximab Abs found in RA patients treated for 3–6 months.
- Fluid-phase RIAs are less sensitive to artefacts due to neo-epitope formation or loss of epitopes which, for example, are known to occur when proteins are fixed to plastic surfaces [27–29].
- The fluid-phase RIA does not favour detection of low-avidity Abs. The high protein concentration of fixed infliximab in the solid-phase RIA favours detection of low-avidity Abs, which may have little clinical implication. This would explain why several sera appeared false positive in solid-phase assays when compared with the results of binding to soluble infliximab only.

Rheumatology key messages

- Assessments of bioavailability and immunogenicity of infliximab may be used to optimize dose regimens and prevent prolonged use of inadequate therapy.
- Functional fluid-phase assays are superior to solid-phase, ELISA-type assays for trough serum infliximab levels and anti-infliximab antibodies.

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