

Comparison of Techniques for Monitoring Infliximab and Antibodies Against Infliximab in Crohn's Disease

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Background: Several techniques are used to measure infliximab (IFX) and anti-IFX antibodies (Abs) in Crohn's disease. The aim of this study was to compare different assays for this purpose.

Methods: Fluid-phase radioimmunoassay (RIA), solid-phase enzyme-linked immunosorbent assay (ELISA), reporter gene assay (RGA), and enzyme immunoassay (EIA; anti-IFX Ab only) were assessed. IFX was added to pooled serum from 13 patients with inactive Crohn's disease to yield concentrations of 0, 1, 3, and 9 µg/mL. Anti-IFX Abs were assessed in 6 patients.

Results: IFX assessments: RIA and RGA had lower limit of detection than ELISA (0.07 µg/mL and 0.13 versus 0.26). Maximal inaccuracies were 39%, 24%, and 23%. Imprecisions (coefficients of variation) were ≤20% within IFX concentrations between 1 and 9 µg/mL. All assays showed linear correlations ($R^2 = 0.97$ – 0.99), but sample concentrations differed by up to 1.55 µg/mL for RIA and RGA, 1.41 µg/mL for ELISA and RIA, and 0.48 µg/mL for ELISA and RGA ($P < 0.05$). Anti-IFX Ab assessments: RGA gave highly reproducible results (coefficients of variation ≤ 7%) compared with all others (24%–26%). All assays had linear correlations ($R^2 = 0.71$ – 0.93), except ELISA versus RGA and EIA. Assays disagreed on anti-IFX Ab titers with mean difference –420 (–1200 to 210) in RGA and EIA, and up to 4500 (–2700 to 11,800) in RIA and RGA.

A contributing factor to these discrepancies was inability of ELISA to detect IgG4 anti-IFX Abs.

Conclusions: Performances of assays for IFX and anti-IFX Abs are comparable. However, IFX concentrations and anti-IFX Ab titers show systematic differences, and in individual patients, only the same assay should be used. Problems may arise when different assays are used to manage therapies in the same patient.

Key Words: inflammatory bowel disease, TNF, ELISA, RIA, RGA (*Ther Drug Monit* 2013;35:530–538)

INTRODUCTION

The chimeric monoclonal anti-tumor necrosis factor α antibody (Ab), infliximab (IFX), is used to induce and maintain remission in patients with moderate-to-severe Crohn's disease (CD).¹ Despite its effectiveness, approximately one third of patients experience primary treatment failure with no response to induction therapy.^{2,3} In addition, a notable fraction of up to about 50% with initial response to IFX induction later lose effect and experience flare of disease during ongoing IFX maintenance therapy.^{2,3} Determining optimal therapy after therapeutic failure is complicated and may impact patient well-being and costs. We have proposed an algorithm for optimizing treatment in patients who lose response to IFX,⁴ which has later been supported by others.^{3,5} The key element is to intervene according to pharmaco-immunological evidence obtained from each individual patient considering 3 principal situations: (1) pharmacokinetic issues with low circulating IFX trough concentrations due to, for example, increased drug turn-over; (2) immunogenicity resulting in generation of specific anti-IFX Abs and increased clearance of functionally active drug; and (3) pharmacodynamic issues with loss of response despite high circulating TNF-neutralizing capacity.^{4,6–10}

In this context, precise and clinically relevant measurements of functionally active IFX and presence of neutralizing anti-IFX Abs in the circulation are warranted. Two principle types of analytical techniques have thus far dominated in clinical investigations: solid-phase enzyme-linked immunosorbent assays (ELISAs) of which a number of different designs have been applied, and solid- and fluid-phase radioimmunoassays (RIAs).^{2,4,10,11} This heterogeneity has made it difficult to compare individual study results, and it has been suggested that reported variations of the clinical importance of IFX and anti-IFX Ab levels may stem from analytical

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incongruence.^{2,10} We therefore aimed at comparing basic analytical properties including level of agreement of commonly used ELISA and RIA techniques for detection of IFX and anti-IFX Abs and to compare these data with those of a recently developed cell-based reporter gene assay (RGA) and a new enzyme immunoassay (EIA) for anti-IFX Ab.^{12,13}

MATERIALS AND METHODS

Patients

Blood samples for IFX analyses were obtained from 13 anti-TNF-naïve patients with CD at inactive stage (Harvey–Bradshaw score < 5 and without fistula activity).^{14,15} Samples for anti-IFX Ab analyses were obtained immediately before IFX infusions from 12 patients with CD (median 14 IFX infusions; range, 5–25) with previously detected anti-IFX Ab measured by RIA (Biomonitor A/S, Copenhagen, Denmark).^{16–18} Samples were screened for anti-IFX Abs using RIA, and 2 samples with low, intermediate, and high anti-IFX Ab titers were selected for further analyses. Concomitant immunosuppressives except steroids within the past 3 months were allowed. The study was approved by the regional ethics committee (H-D-2009-055) and the Danish Data Protection Agency (2009-41-3479). All subjects gave written and oral informed consent.

Sample Preparation

Serum was collected after centrifugation and stored at –80°C. Samples were blinded for patient information, concentration of IFX, and previous concentration of detected anti-IFX Abs. Samples were assessed by the same technician at Biomonitor A/S. Test results were calculated as means of duplicate assessments, and samples were retested if the difference was >20%.

IFX Analyses

Samples for intra-day and between-days IFX analyses were prepared from pools of sera from the 13 study patients. Stock solutions of 10 mg/mL of IFX were prepared by adding 10 mL of sterile distilled water to a vial of 100 mg of IFX (MSD, Whitehouse Station, NJ). IFX was then added to the pooled serum samples to yield final IFX concentrations of 0, 1, 3, and 9 µg/mL for intra-day testings, and 0 and 3 µg/mL for between-days testings. The IFX concentration in each sample was measured 6 times on the same day (intra-day assessment) or once on 6 separate days (between-days assessments). In addition, inter-individual testing was done by adding IFX to each of the 13 patient sera to a final concentration of 0 and 3 µg/mL followed by a single IFX concentration measurement. These data were used to determine the limits of detection for each assay as the mean background activity + 3 SDs, imprecisions as coefficients of variation (CV%), inaccuracies as bias (% of expected concentrations), agreements as difference between mean concentration of the repeated measurements, and correlations between assays.

Anti-IFX Ab Analyses

Intra-day and between-days anti-IFX Ab testings were measured in sera from patients with low, intermediate, or high

anti-IFX Ab titers. Anti-IFX Abs were measured 6 times in each sample to determine intra-day and between-days imprecisions. Correlations and agreements between techniques were evaluated by comparing Ab concentrations (the mean of 6 repeated measurements) measured by each pair of assays.¹⁹ The lowest limits of quantification were chosen as titers obtained at a similar readout point, the latter of which in each technique defined as the mean background activity in medium + 10 SDs; $n = 5$, making $P < 0.01$ for a false-negative readout, and allowing a common unitage for comparisons of the different assays.

Tests for Serum Concentrations of IFX and anti-IFX Abs

Solid-Phase Capture ELISA for IFX

Capture ELISA for IFX was carried out as described by Ternant et al.²⁰ Briefly, 96-well micro titer plates (Maxisorp; Nunc, Roskilde, Denmark) were incubated at 4°C overnight with 100 µL carrier-free *Escherichia coli*-derived recombinant human TNF- α (R&D Systems, Minneapolis, MN) at 0.75 µg/mL in 1 mol/L carbonate–bicarbonate buffer, pH 9.6. After wash with phosphate-buffered saline (PBS), 0.5% Tween 20, each well was blocked with 200 µL of PBS, 1% bovine serum albumin (BSA; Chron Fraction V; Sigma–Aldrich, St. Louis, MO). Before assay, the plates were washed with PBS, 0.05% Tween 20. A calibration curve for IFX concentrations was prepared by adding IFX, serially 2-fold diluted from 40 to 0.62 ng/mL of PBS, 1% BSA. In parallel, serum samples were added at a final concentration of 0.2% and consecutive 2-fold dilutions in PBS, 1% BSA. All samples (100 µL in duplicates) were incubated overnight, washed with PBS, 0.05% Tween 20, and rabbit anti-human IgG/HRP was added (Dako, Glostrup, Denmark) in 100 µL of PBS, 0.5% BSA. After 2 hours, the plates were washed and 100 µL of Tetramethylbenzidine (TMB) One component HRP microwell substrate (BioFX Laboratories, Owings Mills, MD) was added. After 30-minute incubation in the dark, the color reactions were stopped by adding 100 µL of 0.5 mol/L sulfuric acid. Reading was at 450 nm using a Power Wave $\times 340$ (Bio-Tec Instruments, Winooski, VT). Calculations of sample IFX concentrations were extrapolated from a 4-parameter curve fit ($r^2 > 0.95$) of the calibration curve using KC4 software v3.2 (Bio-Tec Instruments). Sample IFX concentrations were expressed as IFX equivalents in micrograms per milliliter.

Solid-Phase Bridging ELISA for anti-IFX Abs

This double-antigen assay was constructed as previously described.^{6,7,21} Briefly, 100 microliters per well of IFX, 0.3 µg/mL of 0.2 M carbonate–bicarbonate buffer, pH 9.4, were incubated overnight. The wells were washed 2 times in PBS, 0.05% Tween 20, and then 200 µL of Superblock in PBS was added (Thermo Scientific, Copenhagen, Denmark) followed by incubation for at least 18 hours. Variable patient serum concentrations were supplemented with pooled normal human serum (NHS) to yield a constant 10% final concentration in 100 µL of PBS, 5 mmol/L of EDTA (Triplex III; Merck Chemicals, Darmstadt, Germany). After overnight incubation, the wells were washed and 100 µL of biotinylated IFX, 20 ng/mL of PBS, 5 mmol/L of EDTA, 0.5% human

serum albumin (HSA) were added (CSL Behring, Lyngby, Denmark). After 2 hours at room temperature, the plates were washed and 100 μ L streptavidin–horseradish peroxidase (Thermo Scientific), 100 ng/mL of PBS, and 0.2% HSA were added to each well. After 1 hour at room temperature, the plates were added TMB and handled as described above.

Fluid-Phase RIA for IFX

IFX concentrations were measured by ^{125} I-TNF- α binding to the IgG fractions of the serum samples.^{16,17,22,23} Briefly, a calibration curve was prepared for each setup using IFX at 0.3–20 ng/mL of PBS, 5 mmol/L of EDTA, 0.5% HSA, and 1% NHS. In parallel, variable concentrations of patient sera were added NHS to a constant serum concentration of 1% in PBS, 5 mmol/L of EDTA, 0.5% HSA, and incubated with 4000–5000 cpm/100 μ L of ^{125} I-TNF- α (Perkin Elmer, Waltham MA). After 2 hours, rabbit anti-human Fc- γ Ab (Dako) was added to each well. Incubation was continued for 2 hours followed by addition of 25 times the sample volume of cold PBS, 5 mmol/L of EDTA, 0.5% HSA. After centrifugation, supernatants were decanted, and pellet activities were measured using a gamma counter (Wizard 1470 Automatic Gamma Counter; Perkin Elmer). Calculations of sample IFX concentrations were extrapolated from 4-parameter curve fits of calibration curves as mentioned above. Sample IFX concentrations were expressed as IFX equivalents in micrograms per milliliter.

Fluid-Phase RIA for anti-IFX Abs

The anti-IFX Ab assay was carried out as previously detailed, using RIA and anti-human lambda light chain Abs to distinguish between free IFX and IFX in complex with any class of lambda-containing human immunoglobulin.^{16,17,22,23} Briefly, variable concentrations of serum samples in PBS, 5 mmol/L of EDTA, were supplemented with NHS to 1% and 4000–5000 cpm/100 μ L of ^{125} I-IFX (Perkin Elmer) was added to each well. After overnight incubation, free and immunoglobulin-bound (any isotype) tracer were separated by precipitation for 4 hours with rabbit anti-human light chain lambda Ab (Dako). Washing and measurements of pellet activities were carried out as described for measurement of IFX by RIA.

RGA for IFX

This assay is based on the iLite TNF-alpha Reporter Cells (Biomonitor, Galway, Ireland), which is a human erythroleukemic K562 cell line transfected with an NFkB-regulated firefly luciferase reporter gene construct.¹³ The cells also contain a Renilla luciferase reporter gene under the control of a constitutive promoter that allows TNF-induced firefly luciferase to be normalized relative to Renilla luciferase expression making results less dependent on the number of viable cells. The engineered cells were cultured in RPMI 1640 with glutamine and 25 mmol/L of HEPES (Biological Industries, Kibbutz Beit Haemek, Israel), 10% fetal calf serum (FCS) (Biosera, Ringmer, East Sussex, United Kingdom), and 5% final human serum concentration. To prepare a calibration curve of residual TNF- α activities, TNF- α (R&D

Systems) were preincubated at 37°C and 5% CO₂ was added with serial 2-fold dilutions of IFX to yield a constant TNF- α concentration of 2 ng/mL and IFX concentration from 100 to 1.5 ng/mL for later cultivation with the reporter cells. After 30 minutes, duplicates of 50 μ L from each preincubated solution were added to 50 μ L of $1\text{--}1.5 \times 10^5$ cells and incubated in white-walled micro 96-well plates (Perkin Elmer) in 5% CO₂. After 3 hours, 80 μ L of Dual-Glo (Promega, Fitchburg, WI) was added to each well followed by reading of firefly luciferase activity in a Victor Light Luminescence Counter (Perkin Elmer). Subsequently, 80 μ L Dual-GloStop&Glo reagent (Promega) was added to each well, and the Renilla luminescence was read. Normalization was made by dividing the firefly with the Renilla luminescence activity in each well. Calculations of the bioactive serum IFX concentrations were carried out on the basis of the linear part of a sigmoidal dose–response (variable slope) fit, $r^2 > 0.95$, of the calibration curve using GraphPad Prism (GraphPad Software, San Diego, CA). Sample concentrations were expressed as equivalents in micrograms per milliliter.

RGA for anti-IFX Abs

Measurements of serum anti-IFX Abs were carried out by co-incubating iLite TNF- α Reporter Cells (Biomonitor) using a slight modification of the method previously described by Lallemand et al.¹³ The reporter cells were cultured in RPMI 1640 with glutamine and 25 mmol/L of HEPES, 10% FCS as above, except that the cells were co-incubated with 2.5% human serum (including patient samples), 40 ng/mL of IFX, and 2 ng/mL TNF- α . Briefly, variable concentrations of patient sera, supplemented with NHS to 20% human serum, were mixed with an equal volume of 320 ng/mL of IFX followed by preincubation at 37°C and 5% CO₂. After 30 minutes, an equal volume of 8 ng/mL TNF- α in RPMI 1640 with glutamine, 25 mM HEPES, and 10% FCS was added to each well. After 30 minutes, 50 μ L of the preincubated samples were added to 50 μ L of cells, and duplicate cell suspensions were then incubated for 3 hours and read for firefly and Renilla luminescence as described.

Solid-Phase EIA for anti-IFX Abs

This EIA measures the binding of IFX to patient IgG preabsorbed to protein G.²⁴ Briefly, Maxisorp 96-well plastic plates (Nunc) were coated with 100 μ L protein G (Thermo Scientific), 25 μ g/mL of 0.2 mol/L carbonate–bicarbonate buffer, pH 9.4. After overnight incubation, the plates were washed with PBS, 0.05% Tween 20, and incubated with Superblock in PBS for at least 18 hours. Variable concentrations of patient sera were tested at a constant human serum concentration of 1% in PBS, 5 mmol/L of EDTA, obtained by supplementing with pooled NHS. After overnight incubation, the wells were washed and added 100 μ L of biotinylated IFX, 20 ng/mL of PBS, 5 mmol/L of EDTA, 0.5% HSA, 4% pooled NHS. After 2 hours, the plates were washed and 100 μ L of 100 ng/mL streptavidin–horseradish peroxidase in PBS, 0.2% HSA, was added to each well. After 1 hour at room temperature, TMB was added to the plates and handled as described above.

Solid-Phase EIA for anti-IFX IgG4

The assay was run as described for the EIA for anti-IFX Abs with the exception of mouse monoclonal Ab to human IgG4 (AbD Serotec; Trikem, Skanderborg, Denmark) being used for coating.

Statistics

The Levene test was used to compare imprecisions of all assays, and in case of significance, each pair of assay was then individually compared. IFX concentrations were first compared by Kruskal–Wallis test and then by Mann–Whitney test in case of significance. Correlations were investigated using linear correlation analysis [Pearson correlation coefficient was used to calculate coefficient of determination (R^2)] followed by linear regression analysis in case of significance (line forced through zero). Nonlinear correlation was calculated in case of lack of a linear correlation and using Spearman rank correlation coefficient (r_s). P values were 2 sided, and $P < 0.05$ was considered significant. Analyses were done in SPSS version 18 (IBM, Somers, NY) and in GraphPad Prism.

RESULTS

IFX Concentration Measurements

Limit of Detection

Limit of detection of IFX was determined using serum from a pool of 13 anti-TNF drug-naïve patients with inactive CD. Tests were repeated the same day and on separate days and also in the individual patient sera. The highest concentration detected under each of these different circumstances was 0.15 $\mu\text{g/mL}$ in ELISA, 0.03 $\mu\text{g/mL}$ in RIA, and 0.06 $\mu\text{g/mL}$ in RGA. Limit of detection was lowest in RIA (0.07 $\mu\text{g/mL}$), followed by RGA (0.13 $\mu\text{g/mL}$), and ELISA (0.26 $\mu\text{g/mL}$).

Imprecision

Imprecision of IFX concentrations between 1 and 9 $\mu\text{g/mL}$ was generally comparable between the assays (Table 1). However, the between-days imprecision of RIA (8%) was significantly lower than that of ELISA (12%, $P < 0.05$) and RGA (20%, $P < 0.01$). Maximal imprecision was 14% in ELISA, 20% in RIA, and 20% in RGA.

Inaccuracy

Inaccuracy describes the degree of closeness of measurements of a quantity to the true value. The inaccuracies of IFX measurements in serum samples with known IFX concentrations between 1 and 9 $\mu\text{g/mL}$ are shown in Table 2. The assays generally underestimated the concentration of IFX in serum. Maximal inaccuracy in each assay was 23% in ELISA, 39% in RIA, and 24% in RGA.

Correlation

As shown in Figure 1, there were highly significant linear correlations between ELISA and RIA [$R^2 = 0.98$ (0.73–1.00), $P = 0.001$], ELISA and RGA [$R^2 = 0.99$ (0.90–1.00), $P < 0.001$], and RIA and RGA [$R^2 = 0.97$ (0.62–1.00), $P = 0.002$]. Furthermore, direct proportionality as defined by a slope (α) of 1 of the linear regression line was observed between ELISA and RGA [$\alpha = 0.97$ (0.87–1.08)] and RIA and RGA [$\alpha = 1.19$ (1.00–1.38)] but not between ELISA and RIA [$\alpha = 0.81$ (0.73–0.89)].

Agreement

As shown in Table 3, there was an overall statistically significant disagreement between the 3 assays when testing sera with different IFX concentrations on the same day, on different days, and in different individuals. This disagreement was also evident when comparing the assays in pair. The maximum difference detected by each pair of assays was 1.41 $\mu\text{g/mL}$ for ELISA and RIA, 0.48 $\mu\text{g/mL}$ for ELISA and RGA, and 1.55 $\mu\text{g/mL}$ for RIA and RGA.

Anti-IFX Ab Titer Measurements

Relative Assay Sensitivity

The absence of a standard reference preparation of anti-IFX Abs makes it difficult to compare detection limits of titer-based assays for Abs. However, when testing 4 sera with high levels of anti-IFX Ab titers, the median titer in RIA was 118-fold higher than that of the RGA, and the ELISA and EIA titers were 24-fold and 11-fold, respectively, higher than the median RGA titer. Hence, the most sensitive assay was RIA, followed by ELISA, EIA, and RGA.

Imprecisions

Intra-day and between-days imprecisions obtained from different patients with low, medium, or high anti-IFX Ab

TABLE 1. Imprecisions of IFX Concentration Measurements

IFX Concentrations, $\mu\text{g/mL}$	Time	CV (%)			P ELISA Versus RIA Versus RGA
		ELISA	RIA	RGA	
1	Intra-day	1	4	4	0.173
3	Intra-day	3	3	3	0.818
3	Between-days	12	8	20	0.016
3	Inter-individual	14	20	13	0.084
9	Intra-day	4	8	8	0.323

P values calculated by the Levene test.

CV, coefficient of variation.

TABLE 2. Inaccuracies of IFX Concentration Measurements

IFX Concentrations, μg/mL	Time	Bias (%)		
		ELISA	RIA	RGA
1	Intra-day	14	−12	−4
3	Intra-day	−16	−29	−19
3	Between-days	−8	−33	−24
3	Inter-individual	0	−5	−14
9	Intra-day	−23	−39	−21

levels are shown in Table 4. Maximal imprecision for each assay was substantially lower in RGA (7%) compared with ELISA, EIA, and RIA (24%–26%). Imprecisions differed significantly between assays (Table 4). When comparing each pair of assays, imprecisions were generally higher in RGA and in EIA compared with ELISA or RIA. RGA exhibited higher imprecision compared with EIA, ELISA, and RIA. RIA was the only assay capable of detecting anti-IFX Ab in all patients including those with low anti-IFX Ab titers, in concordance with RIA being the most sensitive assay.

Correlation

As shown in Figure 2, there was a linear correlation between 4 of the 6 pairs of assays: ELISA and RIA [$R^2 = 0.73$ (0.02–0.97), $P = 0.03$], RIA and RGA [$R^2 = 0.75$ (0.03–0.97), $P = 0.03$], RIA and EIA [$R^2 = 0.71$ (0.01–0.96), $P = 0.04$], and RGA and EIA [$R^2 = 0.93$ (0.47–0.99), $P < 0.01$]. Direct proportionality defined as a slope (α) of 1 was observed between ELISA and RIA [$\alpha = 1.41$ (0.53–2.89)] but not between RIA and RGA [$\alpha = 0.01$ (0.01–0.01)], RIA and EIA [$\alpha = 0.10$ (0.06–0.14)], and RGA and EIA [$\alpha = 10.84$ (8.57–13.12)]. A nonlinear correlation was observed in the remaining 2 of the 6 pairs of assays: ELISA and RGA ($r_s = 0.93$, $P = 0.02$), and ELISA and EIA ($r_s = 0.89$, $P = 0.03$). This was at least partly due to an inability of bridging ELISA to detect monovalent IgG4 Abs against IFX. Thus, 2 of the 6 randomly selected sera contained considerable amounts of IgG4 anti-IFX Abs (Fig. 3, black symbols). ELISA of these sera revealed only low titers—most likely representing non-IgG4 anti-IFX Abs. A low concentration of IgG4 in 1 sample (o) was undetectable in all anti-IFX Ab assays.

Agreement

The inaccuracies of assays for anti-IFX Abs could not be determined because anti-IFX Ab titers in individual sera

were unknown. Instead, agreement between each pair of assays was compared by determining differences in titers with the mean values.¹⁹ Highest agreement was observed between RGA and EIA with a mean titer difference of -500 (-900 to -100). The difference for ELISA and EIA was 1600 (-1800 to 5100), 2100 (-1600 to 5800) for ELISA and RGA, 2400 (-5000 to 200) for ELISA and RIA, 4000 (500 – 7600) for RIA and EIA, and 4500 (600 – 8400) for RIA and RGA.

DISCUSSION

The usefulness and generalizability of previously reported associations of IFX and anti-IFX Abs with clinical response types to IFX is limited by lack of knowledge on how different assays compare.^{4,9}

Assays for IFX

Clinical investigations of IFX concentrations have primarily been carried out with different types of solid-phase ELISAs.^{20,21,25} However, fluid-phase RIA,^{16,17,22,23} cell-based RGA,¹³ and mobility shift assays²⁶ have also been applied. We found that RIA and RGA had considerably lower limit of detection compared with ELISA. Imprecisions detected in all assays were $\leq 20\%$ in the range from 1 to 9 $\mu\text{g/mL}$ and generally comparable between assays at different IFX concentrations. However, between-days imprecision was notably higher in RIA, which may be important in the clinical setting where a high degree of reproducibility over time is required. Maximal inaccuracy of assays was 23% in ELISA, 24% in RGA, and 39% in RIA. Inaccuracies could not be compared statistically, as this is a descriptive parameter of the percentage of deviation of test results from actual concentration. Significant linear correlations were observed between each pair of assays, and IFX concentrations were directly proportional in ELISA and RGA, and in RIA and RGA. Notably, however, assays disagreed on IFX concentrations with a maximum difference of 1.55 $\mu\text{g/mL}$ between RIA and RGA, 1.41 $\mu\text{g/mL}$ between ELISA and RIA, and 0.48 $\mu\text{g/mL}$ between ELISA and RGA. The IFX concentrations were chosen to cover a clinically relevant range based on the previous reportings.^{16–18}

We conclude that ELISA, RIA, and RGA for IFX assessments are comparable with respect to basic analytical parameters. In addition, the fractional change in IFX concentration seems constant between assays implying that observed trends in studies using these assays are in theory equivalent. However, IFX concentrations cannot be directly compared

FIGURE 1. IFX concentration measurements. Mean IFX concentrations measured by ELISA, RIA, and RGA in sera with drug concentrations of 1 $\mu\text{g/mL}$ (6 intra-day measurements per assay), 3 $\mu\text{g/mL}$ (6 intra-day, 6 between-days, and 13 inter-individual measurements per assay), and 9 $\mu\text{g/mL}$ (6 intra-day measurements per assay). Linear regression lines are shown, and added identity lines ($r = 1$) are shown as dashed lines.

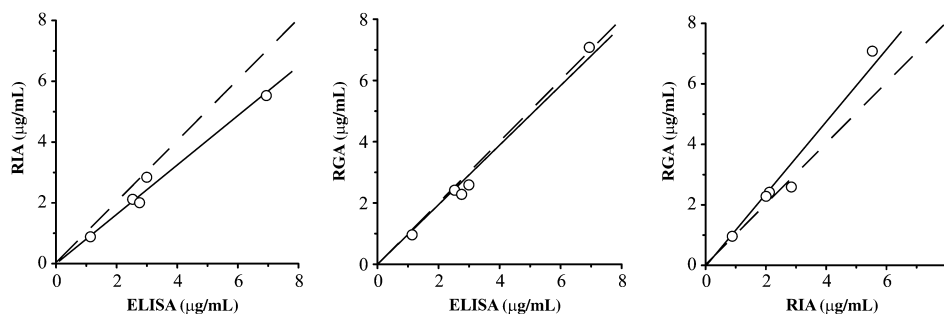


TABLE 3. Assay Agreement on IFX Concentrations

IFX Concentrations, μg/mL	Time	Δ ELISA Versus RIA, μg/mL	Δ ELISA Versus RGA, μg/mL	Δ RIA Versus RGA, μg/mL	All Assays, <i>P</i>
1	Intra-day	0.25	0.17	−0.08	<0.001
3	Intra-day	0.41	0.11	−0.30	0.001
3	Between-days	0.75	0.48	−0.28	0.024
3	Inter-individual	0.15	0.40	0.25	0.008
9	Intra-day	1.41	−0.14	−1.55	0.004

P values calculated by Kruskal–Wallis test.

Δ, Difference.

between assays, and they may vary up to several micrograms per milliliter. The reason for this is unknown but may at least partly be due to the ability of RIA to measure the TNF-binding capacity in fluid phase and the ability of RGA to directly measure the TNF-neutralizing effect of IFX at the cellular TNF receptor level. It should also be noted that factors in individual sera may interfere differently with the binding of IFX to TNF not only in serum samples but also during the assay procedure.

Assays for anti-IFX Abs

Sandwich-type ELISAs for detection of anti-IFX Abs have a major disadvantage because the standard Ab used for detection of anti-IFX Abs may also bind IFX.² Consequently, most clinical studies have applied a bridging-type ELISA in which plated IFX serves as antigen, and biotinylated or otherwise tagged IFX is used to detect anti-IFX Abs bound to the plated IFX.^{6,7,21} RIA,^{16,17,22,23} RGA,¹³ EIA,¹² Western blotting,⁹ chromatography,²⁷ and mobility shift assay²⁶ have also been used to quantify anti-IFX Abs in the clinical setting.

We assessed the basic analytical properties of a bridging ELISA, RIA, RGA, and a recently developed EIA²⁴ and determined anti-IFX Ab titers using a common readout point in all assays to facilitate interassay comparisons. A linear correlation was observed between all pairs of assays, except for ELISA versus RGA and ELISA versus EIA which,

however, exhibited a significant nonlinear correlation. There was also significant disagreement on anti-IFX Ab titers in individual serum samples. Contributing to both issues was the inability of ELISA to quantify functionally monovalent anti-IFX IgG4 Abs, as opposed to RIA, RGA, and EIA. Maximal imprecision was substantially lower in RGA (7%) compared with all others assays (24%–26%). All assays had intra-day imprecisions ≤20%. ELISA, RIA, and EIA had between-days imprecisions >20%, which is unacceptable from an analytical point of view. Only RIA was sensitive enough to detect anti-IFX Abs in all patients, including those with low titers.

We conclude that although ELISA, RIA, RGA, and EIA for detection of anti-IFX Ab titers are comparable with respect to basic analytical properties, the lower sensitivity of RGA and the inability of bridging ELISA to detect anti-IFX IgG4 may have clinical importance. Of note, IgG4 Abs have been shown to constitute up to 89% of anti-IFX IgG Abs in patients treated for prolonged periods of time.^{11,12,23} The fact that RIA detects lambda light chain anti-IFX Ab only, as opposed to the 3 other assays, which detect both lambda and kappa light chain anti-IFX Abs, does not seem to limit the clinical usefulness of this assay. Hence, anti-IFX IgG Abs express kappa and lambda light chains at the same ratio as other IgG molecules, and binding avidities are largely

TABLE 4. Imprecisions of Anti-IFX Ab Titer Measurements

Anti-IFX Ab Titer	Time	CV (%)				<i>P</i>						
		ELISA	RIA	RGA	EIA	All	ELISA Versus RIA	ELISA Versus RGA	ELISA Versus EIA	RIA Versus RGA	RIA Versus EIA	RGA Versus EIA
Low	Intra-day	8	18	*	*	—	<0.001	—	—	—	—	—
Medium	Intra-day	12	20	6	8	0.039	0.110	0.015	0.407	0.082	0.101	<0.001
High	Intra-day	10	9	7	8	0.001	0.053	0.003	0.014	0.007	0.010	0.018
Low	Between-days	*	25	*	17	—	—	—	—	—	0.119	—
Medium	Between-days	26	26	5	24	<0.001	<0.001	0.031	0.926	<0.001	<0.001	0.005
High	Between-days	23	23	3	14	<0.001	0.291	0.015	0.022	<0.001	<0.001	0.009

*Anti-IFX Ab not detected in assay.

P values calculated by the Levene test.

—, Not applicable; CV, coefficient of variation.

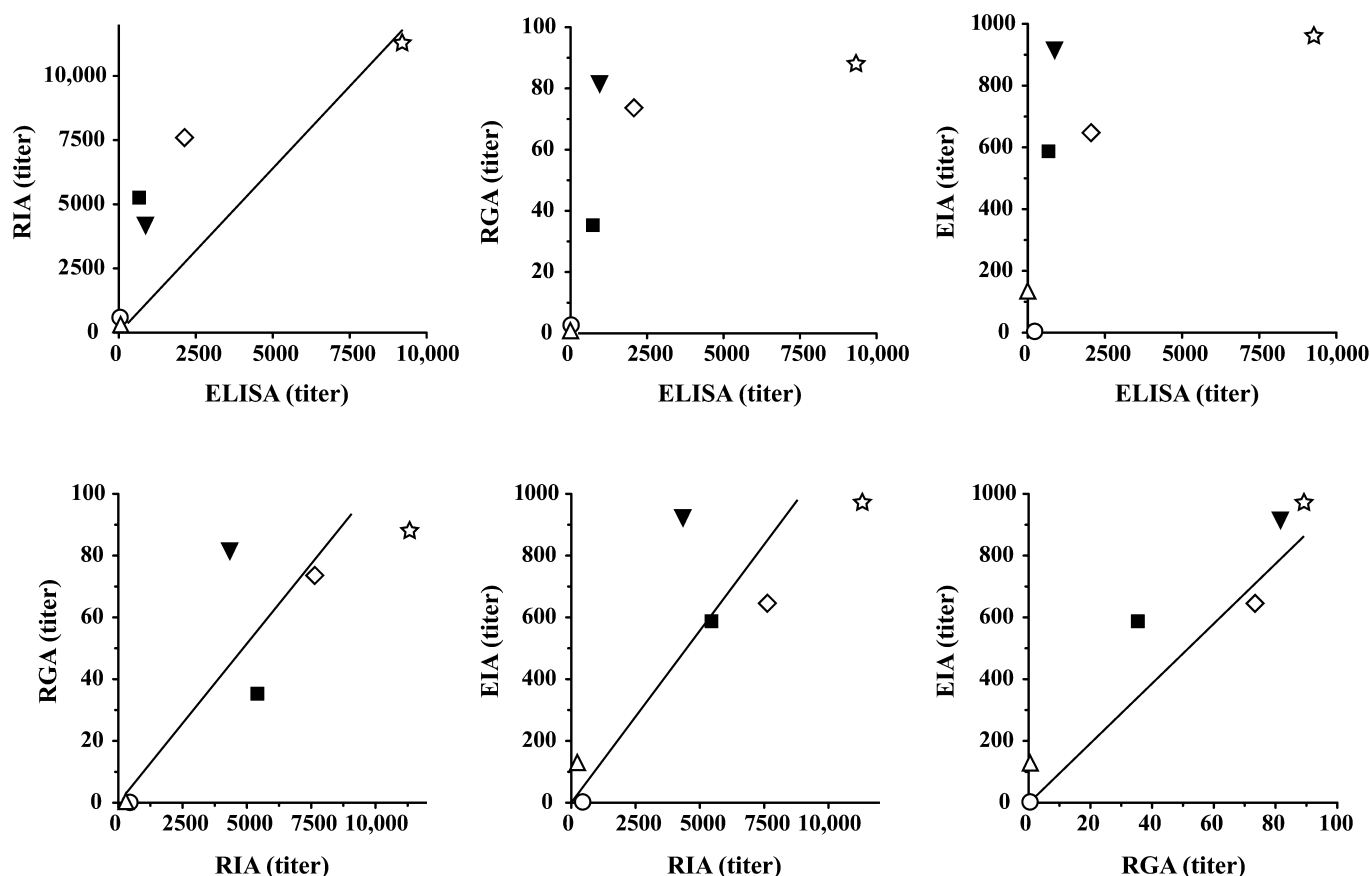


FIGURE 2. Anti-IFX Ab titer measurements. Anti-IFX Ab titers measured by ELISA, RIA, RGA, and EIA. Titers are means of 6 repeat analyses in each of 6 different patient sera (unique symbols). Linear regression lines are shown.

independent of the light chain isotype.^{22–24} Although our findings indicate that observed trends in studies using different assays for anti-IFX Abs may be equivalent, anti-IFX Ab titers cannot be directly compared between assays. In addition, disagreement on anti-IFX Ab titers may result from different susceptibilities of assays to neopeptide formation and/or epitope masking during testing, differences in detecting low-affinity binding Abs, and different degrees of interference

by sample viscosity, pH, and salt levels. Factors in sera such as heterophilic Abs, rheumatoid factors, and activated complement components may also interfere with assays to variable degrees, as will the presence of the drug itself.^{2,4,11,28} These concerns are particularly relevant when monitoring individual patients, where severe consequences may result if therapeutic decisions are made on the basis of an assay that does not reflect the situation in vivo.

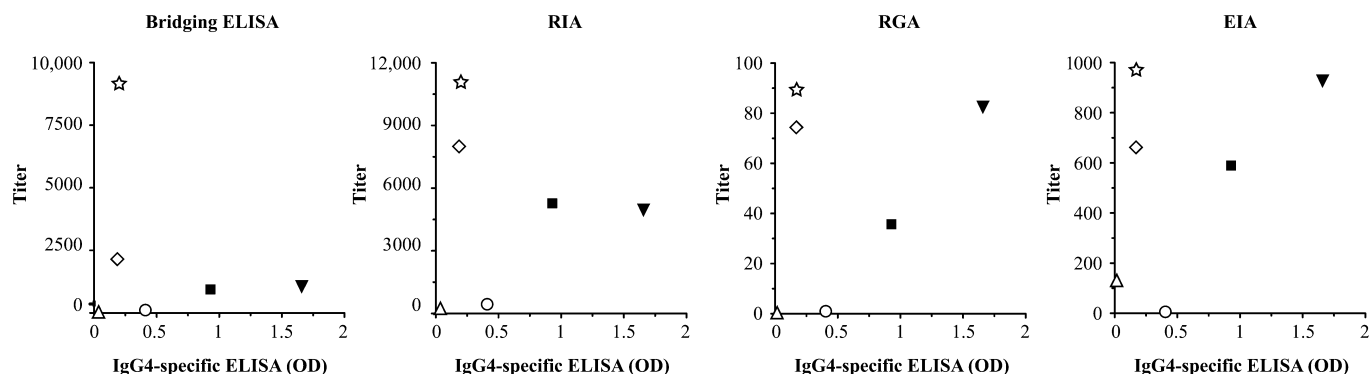


FIGURE 3. Anti-IFX IgG4 Ab titer measurements. Anti-IFX IgG4 Abs in the 6 sera visualized in Figure 2 compared with total anti-IFX Ab titers measured by ELISA, RIA, RGA, and EIA. Same patient-specific symbols as in Figure 2, and sera with high IgG4 fractions shown with solid symbols.

Study Limitations

ELISAs for IFX and anti-IFX Abs were designed in accordance with the information provided in previous publications, and it is possible that other subtypes of ELISAs may have different characteristics.^{6,7,20,21} The number of individual patients and the number of repeat measurements in this study were generally low, and this was the case also when determining limits of detection. This study did not assess the ability of IFX assays to measure biologically active TNF-neutralizing drug concentrations or the ability of anti-IFX Ab assays to measure functionally active, IFX-neutralizing anti-IFX Ab concentrations. The individual assay's ability to accurately detect anti-IFX Abs in the presence of IFX was not determined here. However, it has previously been established that bridging ELISA is unable to detect anti-IFX Abs in the presence of IFX, thereby rendering test results from about half of the patients in clinical trials inconclusive.^{6,7,29,30} Capture ELISA using anti-human lambda Ab for detection may perform better.^{9,31} However, RIA and RGA measure anti-IFX Abs also in the presence of moderate concentrations of IFX as seen in trough serum samples.^{4,13,22} Assay characteristics in patients with active CD were not assessed here and may in theory be different. The true anti-IFX Ab concentrations in individual patient sera were unknown, and the accuracies of detected anti-IFX Ab concentrations could therefore not be compared. As there is no gold standard anti-IFX Ab assay, selection of anti-IFX Ab-positive samples for evaluations were based on the initial screening by RIA which may have biased the study results. Comparison of assay performances to aid in optimizing IFX therapy in individual patients was not investigated here and remains yet unresolved.

It should be noted that ELISA, EIA, and RGA have advantages of being relatively easy to use in local laboratories, whereas RIA requires more advanced laboratory facilities. It is also noteworthy that RGA is a cell-based assay, which does not have the same characteristics as the binding assays. This is revealed in Figure 2 and in the anti-IFX Ab sensitivity measurements, where titers obtained in RGA vary by 1–2 orders of magnitude from those of the binding assays despite a common readout point for titer determination. The RGA detects drug activity, not drug (protein) concentrations as do the binding assays, and its test outcome includes other factors that may interfere with TNF-mediated cellular effects in vivo, for example, soluble TNF receptors. Most importantly, however, RGA only measures functionally active antidrug Abs, that is, Ab with epitope characteristics and sufficient affinities to interfere with drug-induced TNF neutralization at the cellular level.

CONCLUSIONS

In patients with CD, basic analytical properties of commonly used techniques for detection of IFX and anti-IFX Abs are comparable, and a high degree of correlation of the different assays indicate that previously observed trends in cohort studies are likely to be similar between assays. However, serum factors and/or matrix effects interfere differently in assays, and problems may occur when data are used to manage therapies in individual patients. As assays disagree with respect to concentrations and abilities to detect IgG4 Abs, associations with clinical response types and

determinations of cut-off concentrations and therapeutic intervals for clinically relevant concentrations of IFX and anti-IFX Abs need to be established for each type of assay. Until such studies have ascertained, we recommend using assays that reflect patient conditions as closely as possible; that is, assays where assessments take place in fluid-phase, where all anti-IFX IgG isotypes and, optimally, other Ab classes are quantified for functional consequences.

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