

Subset Analysis of Patients Experiencing Clinical Events of a Potentially Immunogenic Nature in the Pivotal Clinical Trials of Tocilizumab for Rheumatoid Arthritis: Evaluation of an Antidrug Antibody ELISA Using Clinical Adverse Event–Driven Immunogenicity Testing

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ABSTRACT

Background: Tocilizumab (TCZ) is a recombinant humanized monoclonal antibody directed against the interleukin-6 receptor. In Europe, TCZ is approved for use in combination with methotrexate in the treatment of adult patients with moderate to severe rheumatoid arthritis (RA) who have failed to respond to or were unable to tolerate previous therapy with one or more disease-modifying antirheumatic drugs or tumor necrosis factor (TNF) antagonists; in the United States, it is approved for the treatment of adult patients with moderate to severe active RA who have failed to respond to one or more TNF antagonists. As part of the Phase III clinical development program, the immunogenicity of TCZ was evaluated using a bridging ELISA; however, this assay is considered limited in detecting low-affinity or immunoglobulin G4 subisotype antidrug antibodies (ADAs).

Objective: This study assessed the validity of the ELISA for detecting anti-TCZ ADAs by using complementary bioanalytic assays to test samples from a subgroup of patients with clinical adverse events (AEs) of a potentially immunogenic nature, who were considered highly likely to have ADAs. The goal was to determine whether use of these additional assays led to detection of ADAs not found on the ELISA, thus minimizing the risk of false-negative results.

Methods: The Phase III program for TCZ consisted of 5 core studies in which adult patients with RA received either TCZ 4 or 8 mg/kg IV or control every 4 weeks, with or without concomitant antirheumatic therapy. Blood samples obtained at baseline and at regular in-

tervals thereafter were tested using the ELISA for ADA screening and confirmation. Regardless of the results on ADA screening, samples from patients who developed clinical AEs of a potentially immunogenic nature (ie, falling within predefined system organ classes, occurring during or within 24 hours of TCZ infusion, considered related to TCZ therapy, or leading to study withdrawal) were subjected to additional testing with a surface plasmon resonance (SPR) assay for isotyping and epitope localization and a standard ImmunoCAP immunoglobulin E (IgE) assay made specific for TCZ.

Results: The 5 core studies and their open-label, long-term extension studies enrolled a total of 4199 adult patients with RA (82.1% female; 74.0% white; mean age, 52.0 years [range, 18–89 years]; mean weight, 73.4 kg [range, 35–150 kg]); 2928 patients received TCZ and 1271 received control. Of the 2816 samples from TCZ-treated patients tested, 64 (2.3%) had samples that tested positive at least once on the ELISA screening and confirmation assay, 48 (75.0%) of them at baseline. A clinical AE of a potentially immunogenic nature occurred during TCZ treatment in 21 patients, 8 of whom had an anaphylactic reaction. Eleven of the samples from

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these 21 patients had tested negative for ADAs on the screening ELISA. Only 1 of these 11 patients tested positive for ADAs on both additional assays; all others tested negative. The results of the ELISA, SPR, and IgE assays were consistent for 16 of 18 tested patients (88.9%) who provided data on at least 2 of the 3 assays.

Conclusions: Based on the findings of this analysis in a relevant patient population, the bridging-type screening and confirmation ELISA was a valid method of detecting anti-TCZ ADAs. Immunogenicity testing of samples from patients with clinical AEs of a potentially immunogenic nature using assays complementary to the ELISA added valuable information about the incidence and character of ADAs. (*Clin Ther.* 2010;32:1597–1609) © 2010 Excerpta Medica Inc.

Key words: antidrug antibody, ADA, immunogenicity, therapeutic antibody, ELISA, adverse event.

INTRODUCTION

As a class, monoclonal antibodies have become important in the treatment of a variety of diseases, and >200 candidate antibody agents are currently in clinical development.¹ Phage display, transgenic mouse, and other technology platforms have been used to create fully human antibodies.² Despite the human nature of therapeutic proteins, immunogenic reactions against biologic products, including therapeutic antibodies, have been reported.³ Such immunogenic reactions need to be monitored during clinical studies, as they have the potential to lead to serious adverse events (SAEs), including hypersensitivity or autoimmune reactions and/or loss of efficacy due to altered pharmacokinetics.³

A number of techniques have been developed for the detection and measurement of antidrug antibodies (ADAs), including ELISAs, electrochemiluminescence immunoassays, radioimmunoprecipitation assays, surface plasmon resonance (SPR) assays, and bioassays.⁴ Each of these assay types has its advantages and limitations.⁵ Expert consensus suggests that immunogenicity testing should consist of at least 2 stages. Stage 1 involves initial screening for ADAs and confirmation of ADA specificity; in the presence of a confirmed positive result, stage 2 involves ADA characterization.³ ADAs may be characterized by their potential neutralizing activity, immunoglobulin isotype (IgG, IgM, IgA, or IgE), and epitope recognition.^{3,4,6}

Tocilizumab (TCZ) is a recombinant humanized monoclonal antibody directed against the interleukin-6

receptor. It was initially approved in Japan in 2005 as a therapy for Castleman disease. TCZ has been reported to be effective in clinical trials in patients with rheumatoid arthritis (RA)⁷ and was approved by the European Commission in January 2009 for use in combination with methotrexate (MTX) for the treatment of adult patients with moderate to severe RA who had failed to respond adequately to or were unable to tolerate previous therapy with one or more disease-modifying antirheumatic drugs (DMARDs) or tumor necrosis factor antagonists (anti-TNFs). It was approved in January 2010 by the US Food and Drug Administration for the treatment of adult patients with moderate to severe active RA who had failed to respond adequately to one or more anti-TNF agents.

Testing for potential immunogenicity in the Phase III clinical trials of TCZ was conducted according to the typical 2-step approach. An ELISA screening assay with a bridging format was selected and developed for screening and specificity confirmation of anti-TCZ ADAs based on the rationale that it provided high throughput, had high specificity, and was theoretically capable of detecting all isotype responses.^{4–6} Secondary assays were used to characterize the neutralizing capacity of ADAs, their immunoglobulin isotype, and their binding epitope.

Because the bridging ELISA is considered limited in detecting low-affinity ADAs due to repeated washing and sample incubation steps and may miss ADAs of the IgG4 subclass,⁸ an innovative AE-driven immunogenicity testing approach was introduced into the Phase III clinical development program for TCZ. Samples from patients with AEs potentially mediated through immunologic mechanisms were tested for ADAs using 2 assays complementary to the ELISA: an SPR assay for isotyping and epitope localization⁹ and an ImmunoCAP IgE assay.¹⁰ Although this approach is not specifically required by regulatory agencies, expert consensus recommends the development of testing strategies that provide valid assessments of antibody responses in clinical trials.³

The SPR assay is more sensitive than the ELISA in detecting lower-affinity ADAs, but is less sensitive in detecting the highest-affinity ADAs.^{8,9} Data provided by the IgE immunoassay should allow correlation of anaphylactic and type 1 hypersensitivity reactions with the presence of IgE ADAs, as has been reported for the anti-TNF antibody infliximab¹⁰ and the therapeutic antibody cetuximab.¹¹ However, hypersensitivity reac-

tions are not necessarily strongly correlated with the formation of IgE ADAs; of 25 cetuximab-treated patients with hypersensitivity reactions, only 17 (68.0%) had IgE antibodies against cetuximab.¹¹ Furthermore, IgE antibodies against cetuximab were detected in 20.8% of control subjects in some areas of the United States.¹¹ Similarly, of 11 infliximab-treated patients with hypersensitivity reactions, 8 (72.7%) had nonisotype-specific ADAs.¹⁰

The present study was undertaken to assess the validity of the screening and confirmation ELISA in detecting ADAs within the context of the Phase III development program for TCZ. Complementary bio-analytic assays were used to analyze ADAs in samples from RA patients who experienced a clinical AE of a potentially immunogenic nature and thus had a high probability of having ADAs.

MATERIALS AND METHODS

Pivotal Clinical Trials

The 2-year LITHE (Tocilizumab Safety and the Prevention of Structural Joint Damage; ClinicalTrials.gov identifier: NCT00106535)^{12,13} trial began in January 2005, with planned analyses at 24 and 52 weeks; its 3-year open-label extension study is ongoing. This trial was conducted in South Africa, North and South America, Australia, Asia, and Europe. RADIATE¹⁴ (Rheumatoid Arthritis Study in Anti-TNF Failures; ClinicalTrials.gov identifier: NCT00106522) was conducted from March 2005 to November 2007 in North America, Australia, and Western Europe. OPTION¹⁵ (Tocilizumab Pivotal Trial in Methotrexate Inadequate Responders; ClinicalTrials.gov identifier: NCT00106548) was conducted from March 2005 to October 2007 in Argentina, Brazil, Canada, Europe, Israel, Mexico, Singapore, and Thailand. The TOWARD¹⁶ (Tocilizumab in Combination With Traditional DMARD Therapy; ClinicalTrials.gov identifier: NCT00106574) and AMBITION¹⁷ (Actemra Versus Methotrexate Double-Blind Investigative Trial in Monotherapy; ClinicalTrials.gov identifier: NCT00109408) studies were conducted from March/April 2005 to December 2007 in South Africa, North and South America, Asia, Australia, and Europe. The AMBITION study included a transition phase, in which patients who were responding well to their blinded treatment regimen either continued blinded treatment or entered the open-label extension study. GROWTH95 and GROWTH96 were long-term, open-label extension studies: patients from OPTION entered GROWTH95,

and patients from RADIATE, TOWARD, and AMBITION entered GROWTH96.¹⁸ Table I provides an overview of the 5 pivotal clinical trials.

Data from the 5 pivotal trials and their open-label extensions were combined in a long-term analysis.¹⁸ The combined safety population included a total of 4199 patients with RA who were treated with TCZ 4 or 8 mg/kg IV every 4 weeks (administered as a 100-mL solution infused over 1 hour) or placebo (administered as a solution of 5% sucrose and 0.005% polysorbate 80 in water), with or without concomitant antirheumatic therapy (MTX or another DMARD). Assessment of the cumulative safety profile of TCZ in the present analysis was based on the TCZ all-exposure population, which included those from the LITHE, RADIATE, OPTION, TOWARD, and AMBITION studies who received at least one dose of TCZ, regardless of whether they entered one of the long-term extension studies. For patients who received TCZ in one of the core studies and continued into one of the extension studies, data were included from the first dose of TCZ in the core study. For patients who received placebo or active control in one of the core studies and continued into one of the extension studies, data were included from the first dose of TCZ in the extension study.

All study sites received written approval from their institutional review boards. Study participants were enrolled after providing written informed consent, which included consent for immunogenicity testing. The studies were conducted in conformity with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonisation (ICH) guidelines for Good Clinical Practice, and in accordance with the applicable local laws and regulations.

Blood Sampling and Analysis

Blood samples for immunogenicity testing were collected at baseline (week 0); at weeks 4, 8, 12, and 24 in the 24-week and 24-month core studies; every 12 weeks in the AMBITION transition phase and the GROWTH95 extension study; and every 24 weeks in the GROWTH96 extension study. If possible, samples were also collected at the time of study withdrawal.

Venous blood samples (3.2 mL) were drawn into an anticoagulant-free collection tube and stored at room temperature for ≥ 30 minutes to allow clotting. Within 60 minutes after blood collection, serum was separated by centrifugation at 1500g at 4°C for 10 minutes. Two aliquots of ≥ 0.5 mL each were transferred to

Table I. Overview of the pivotal Phase III clinical trials of tocilizumab (TCZ) in patients with rheumatoid arthritis (RA).

Authors	Study Population	Duration of Therapy	Regimens
Kremer et al ^{12,13} (LITHE)	Moderate to severe active RA with MTX failure	24 mo	TCZ 4 or 8 mg/kg IV q4wk (n = 798) Placebo (n = 392) Both with MTX 10–25 mg/wk
Emery et al ¹⁴ (RADIATE)	Moderate to severe active RA with anti-TNF failure and stable MTX therapy	24 wk	TCZ 4 or 8 mg/kg IV q4wk (n = 338) Placebo (n = 160) Both with MTX 10–25 mg/wk
Smolen et al ¹⁵ (OPTION)	Moderate to severe active RA with MTX failure	24 wk	TCZ 4 or 8 mg/kg IV q4wk (n = 418) Placebo (n = 204) Both with MTX 10–25 mg/wk
Genovese et al ¹⁶ (TOWARD)	Moderate to severe active RA with DMARD failure	24 wk	TCZ 8 mg/kg IV q4wk (n = 802) Placebo (n = 414) Both with DMARD(s)
Jones et al ¹⁷ (AMBITION)	Moderate to severe active RA, MTX naive	24 wk	TCZ 8 mg/kg IV q4wk (n = 288) MTX 7.5–20 mg/wk (n = 284) Placebo (for double-dummy design)
AMBITION transition substudy ¹⁷	Moderate to severe active RA, MTX naive	Maximum, 24 wk	TCZ 8 mg/kg IV q4wk (n = 54) MTX 7.5–20 mg/wk (n = 46) Placebo (for double-dummy design)
GROWTH95/ GROWTH96 ¹⁸	Patients from OPTION entered GROWTH95; patients from RADIATE, TOWARD, and AMBITION entered GROWTH96	5-y Open-label extension studies	TCZ 8 mg/kg IV q4wk, with/without DMARD(s) (n = 2439)

LITHE = Tocilizumab Safety and the Prevention of Structural Joint Damage; MTX = methotrexate; RADIATE = Rheumatoid Arthritis Study in Anti-TNF Failures; TNF = tumor necrosis factor; OPTION = Tocilizumab Pivotal Trial in Methotrexate Inadequate Responders; TOWARD = Tocilizumab in Combination With Traditional DMARD Therapy; DMARD = disease-modifying antirheumatic drug; AMBITION = Actemra Versus Methotrexate Double-Blind Investigative Trial in Monotherapy.

polypropylene storage tubes and stored frozen at -70°C or -20°C (depending on the site) for no longer than 4 weeks until shipped to a central laboratory for analysis. One aliquot was used for the screening and confirmation ELISA, and the other was used for the characterization assays. To guarantee masking of laboratory personnel, samples were randomly positioned in the containers sent to the laboratories.

ELISA was performed at Huntingdon Life Sciences, Ltd. (Huntingdon, United Kingdom) for the OPTION and TOWARD trials, and at SRL, Inc. (Tokyo, Japan) for the LITHE, RADIATE, and AMBITION trials. All

SPR measurements were performed at Roche Diagnostics GmbH (Penzberg, Germany), and all IgE measurements were performed at SRL, Inc.

Bioanalytic Immunogenicity Testing Strategy

Bioanalytic immunogenicity testing for TCZ followed the standard 2-tiered approach (Figure). In stage 1, all patient samples were subjected to routine ELISA screening for ADAs. Those that tested positive were then subjected to the confirmation ELISA. A positive result on both assays triggered further analysis of the samples in stage 2, which involved 4 assays: a neutralization

ELISA to evaluate the potential of ADAs to block binding of TCZ to soluble interleukin-6 receptors; an SPR assay to characterize the ADA isotype and roughly localize the epitope on TCZ against which ADAs were directed¹⁹; an ImmunoCAP IgE assay to specifically analyze ADAs of the IgE isotype²⁰; and a Fab assay to detect binding to the antigen-binding fragment (Fab) of TCZ (this was not included in the AE-driven analysis).

Adverse Event–Driven Immunogenicity Testing Strategy

In addition to routine bioanalytic immunogenicity testing, samples from any patients who experienced AEs of a potentially immunogenic nature (as reported by the principal investigators) during or within 24 hours after the infusion were subjected to stage 2 testing with the SPR and IgE-specific assays at baseline and at

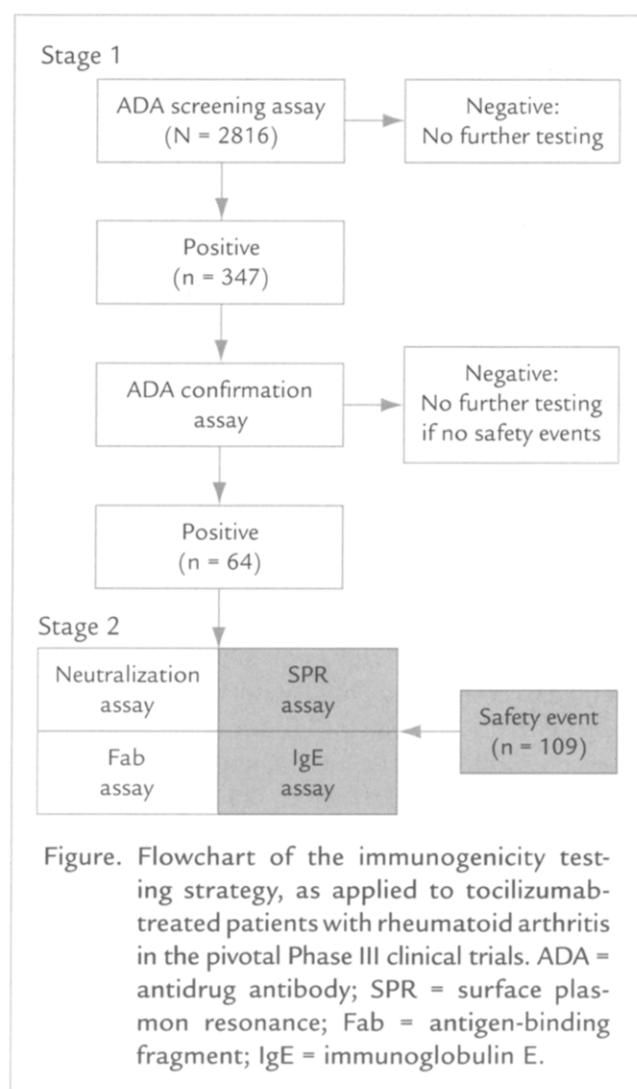
weeks 4, 8, 12, and 24, regardless of the results on the screening and confirmation ELISA. To qualify for stage 2 testing, the infusion-related AE had to be either included on a predefined list of Medical Dictionary for Regulatory Activities (MedDRA) preferred terms for events of a potentially immunogenic nature/potential hypersensitivity reaction or lead to discontinuation/modification of study medication, as documented in the database. Infusion-related AEs were further characterized by whether they met ICH criteria for an SAE (ie, be fatal or life threatening, require inpatient hospitalization or prolongation of existing hospitalization, result in persistent or significant disability); led to initiation of or a change in concomitant medications classified as analgesics, antihistamines, and/or corticosteroids; or reached moderate to severe intensity. Infusion-related AEs were reported according to the timing of their occurrence (during the infusion or within 24 hours afterward). Subsequently, the following algorithm was applied to capture infusion-related AEs: the event had to occur during or within 24 hours after TCZ infusion, be classified as related to TCZ treatment by the investigator, and lead to study withdrawal.

In patients with positive results on the screening and confirmation assays at any time point, samples were also subjected to a neutralizing assay that was not part of the AE-driven analysis. In patients with a sample that tested positive on the screening assay, tested positive or was missing on the confirmation assay, and tested positive or was missing on the SPR or neutralizing assay, the patient was categorized as positive for anti-TCZ antibodies. If the result of the confirmation assay was positive and the sample tested negative on the SPR or neutralizing assay, the patient was still considered ADA positive. A patient was also considered positive for anti-TCZ antibodies if the sample was negative or missing on the screening and confirmation assays but positive on the SPR assay, and the patient experienced an AE of a potentially immunogenic nature within 24 hours after TCZ infusion.

Immunogenicity Assays

Screening and Confirmation Assays

The anti-TCZ antibody screening assay employed a bridging ELISA method,^{21,22} with an additional competitive displacement step as the confirmation assay. The ELISA employed biotinylated TCZ (TCZ-Bi) immobilized on streptavidin-coated microtiter plates (SAMTPs) for the capture of anti-TCZ antibodies com-



plexed with preincubated digoxigenin-labeled TCZ (TCZ-DIG). The bound complex of anti-TCZ antibody and TCZ-DIG was detected based on the reaction between a peroxidase-conjugated anti-DIG antibody and its substrate ABTS and subsequent photometric readout.

The assay was performed at room temperature. In the first step, TCZ-Bi was bound to SA-MTPs at a concentration of 0.5 µg/mL by incubating 100 µL on a shaker at 400 rpm for 1 hour. Before adding the preincubation solution to the SA-MTPs, the excess unbound TCZ-Bi was removed by washing 3 times. In parallel with the coating procedure, preincubation of standards and samples was performed in duplicate in a separate uncoated 96-well plate. The samples and standards were diluted (1:10) in the wells with 10% serum matrix to a volume of 75 µL and mixed with the same volume of TCZ-DIG, starting the 1-hour preincubation. The TCZ-Bi-coated SA-MTPs were loaded with the preincubation solutions by transferring 100 µL from each well of the preincubation plate to the wells of the coated MTP and incubated on a shaker at 400 rpm for 1 hour. After washing, polyclonal anti-DIG horseradish peroxidase (HRP) conjugate in a volume of 100 µL (100 mU/mL) was added to the wells and incubated on a shaker for 1 hour. After washing, the HRP-catalyzed colorigenic reaction was initiated by adding 100 µL ABTS solution. When the maximum optical density (OD) was ~2.0, usually within 20 to 30 minutes, the signal of the color reaction was measured by an ELISA reader at a wavelength of 405 nm (reference, 490 nm). The same assay was performed in the presence of the confirmation reagents, with simultaneous measurement without the confirmation reagents. The obtained OD data were used for generating the standard calibration curve by nonlinear 4-parameter regression curve fitting according to the Wiemer-Rodbard method for calculating the sample concentration.²³

The cutoff points for the test results were set at the 95% CIs of the assay signals (OD) from multiple analyses of human blank serum samples from healthy volunteers and patients with RA.⁵ A screening test result was considered positive at a value above the cutoff. A decrease in absorbance of >20% relative to the unspiked sample indicated a positive result. The screening cutoff was determined at 61.4 ng/mL of reference antibody. Intra-assay and interassay accuracy were 84.8% to 93.1% and 91.3% to 92.2%, respectively. The corresponding values for intra-assay and interassay precision were 1.8% to 2.0% and 6.8% to 8.0%. The accuracy of the ELISA

was defined by the extent to which the results of the assay agreed with the true values. Accuracy was determined by comparing measured concentrations of the rabbit polyclonal anti-TCZ-positive control standard spiked into human serum with nominal concentrations of anti-TCZ. High-concentration (360 ng equivalents/mL) and low-concentration (60 ng equivalents/mL) positive control standards were analyzed in 6 aliquots of each positive control standard measured in duplicate to determine intra-assay accuracy and in 3 aliquots of each positive control standard measured in duplicate to determine interassay accuracy.

SPR Assay

The SPR assay was used to detect ADAs, to determine the isotype of the ADA (IgG, IgM, or IgE), and to localize the epitope (Fab or constant fragment [Fc]) on TCZ. The assay setup made efficient use of the 4 parallel flow cells on a single biosensor chip by immobilization of full-length antibody and its Fc and Fab fragments for differential binding analysis of ADAs. For quality-control (QC) and calibration standards, isotype-specific ADA conjugates were prepared from rabbit polyclonal anti-TCZ antibodies covalently linked to a purified human polyclonal IgG, IgM, or IgE preparation.¹⁹ An evaluation of the SPR assay for determining ADAs and characterizing their isotype and epitope localization has recently been published.¹⁹

IgE Assay

Detection of anti-TCZ IgE antibodies in human serum was performed using a commercially available ImmunoCAP assay system (UniCAP Specific IgE assay and UniCAP 1000 instrument, Sweden Diagnostics Inc., Kalamazoo, Michigan). This system employs allergens covalently bound to a cellulose carrier contained in a reaction capsule (CAP). IgE antibodies that have bound to antigen on the solid phase are detected using a secondary enzyme-labeled anti-IgE antibody. The analytic precision and accuracy of the immunoassay for specific IgE have been reported elsewhere.²⁰ The fully automated assay was performed according to the instructions using reagents purchased from Phadia AB (Uppsala, Sweden). The assay was validated in anti-TCZ IgE-positive human control sera obtained from 2 patients during clinical studies of TCZ.

The assay employed CAPs with bound allergen (TCZ), calibration CAPs, and CAPs with bound house dust mite (*Dermatophagoides pteronyssinus*) allergen (for QC

purposes). Study samples were incubated with the TCZ CAPs, calibration samples with the calibration CAPs, and QC samples with the house dust mite CAPs. The CAPs were allowed to react with any specific IgE antibody in the serum, and excess serum components were removed by washing. The IgE antibody bound to the ImmunoCAP allergens was reacted with β -D-galactosidase-labeled human anti-human IgE antibody. Subsequent addition of a fluorogenic substrate (4-methylumbelliferyl- β -D-galactopyranoside) led to the generation of fluorescent product. The reaction was terminated and the antibody concentration estimated by comparison with the intensity of fluorescence of the calibration samples. The assay had a lower limit of quantitation of 0.35 unit of assay (UA)/mL, a measurement range of 0.35–100 UA/mL, and an interassay precision of 10.3%.

RESULTS

Analytic Population

A total of 2928 patients receiving TCZ and 1271 receiving placebo or active control were included in the core studies with a 24-week observation period. These patients had a mean age of 52.0 years (range, 18–89 years) and a mean weight of 73.4 kg (range, 35–150 kg); they were predominantly female (82.1%) and white (74.0%). There were 5 deaths each in the TCZ and control arms. In the combined TCZ arms, 178 SAEs occurred in 152 patients; in the combined control arms, 89 SAEs occurred in 70 patients. Previous anti-TNF treatment was reported in 23.4% of TCZ recipients and 24.0% of control recipients.

Immunogenicity Screening and Confirmation Assays

The immunogenicity screening assay was performed on samples from 2816 patients in the TCZ arms and 702 patients in the control arms. Of all patients tested in the TCZ arms, 347 (12.3%) had a sample that was positive on the screening assay at some time during the study and was hence tested on the confirmation assay. Of all patients tested in the control arms, 94 (13.4%) had a sample that was positive on the screening assay at some time during the study and was tested on the confirmation assay. In the TCZ arms, 64 patients (2.3%) had samples that were positive at least once on both the screening and confirmation assays. In the control arms, 23 patients (3.3%) had samples that were positive at least once on both the screening and confirmation assays.

Among the 64 TCZ recipients with a positive result on both the screening and confirmation assays, 48 (75.0%) had a positive result on the screening and confirmation assays at baseline (before initiation of TCZ). Among the 23 control recipients with a positive result on both the screening and confirmation assays, 17 (73.9%) had a positive result at baseline. Of the patients who were ADA positive at baseline, 15.6% of TCZ recipients and 15.0% of control recipients had received previous anti-TNF medication. The results of the screening and confirmation assays are summarized in Table II.

Patients were tested for immunogenicity in the extension studies as well as in the core studies. Of 54 patients continuing blinded TCZ treatment for 12 or 24 additional weeks in the AMBITION transition phase, 52 provided samples for testing on the screening assay. None of the results were positive. GROWTH95 and GROWTH96 included 2439 patients who had been treated with TCZ or control for 24 weeks in one of the core studies and were exposed to additional treatment with TCZ for up to 5 years. During the initial 24-week observation period, 54 of 2315 tested patients (2.3%) had positive results on the screening and confirmation assays at some time point; however, 39 of them had already had positive results on these assays at baseline. Thirty of the 2315 screened patients (1.3%) had specific anti-TCZ antibodies; thus, the frequency of ADA-positive patients in the extension studies was similar to that in the core studies (0.6%).

Adverse Event–Driven Immunogenicity Testing

Application of the clinical AE-driven algorithm for immunogenicity testing using the list of MedDRA preferred terms identified 109 TCZ-treated patients in the core studies and 234 TCZ-treated patients in the long-term extension studies who qualified for additional testing on the SPR and IgE assays. For comparison, 58 (3.7%) of the 1555 control patients developed infusion-related reactions that fit the MedDRA preferred terms.

The TCZ all-exposure population consisted of 4009 patients. Application of the additional criterion identified 21 patients (0.5%) with treatment-related AEs that led to study withdrawal. Nine of these had AEs recorded as an anaphylactic reaction (including the terms *anaphylactic reaction*, *anaphylactic shock*, *anaphylactic transfusion reaction*, *anaphylactoid reaction*, *anaphylactoid shock*, *shock*, *type I hypersensitivity*, *circulatory collapse*, *first use syndrome*, and *Kounis*

Table II. Results of immunogenicity screening and confirmation assays in samples from the 5 pivotal Phase III studies of tocilizumab (TCZ).

Variable	TCZ 4 mg/kg + MTX (n = 1058)	TCZ 8 mg/kg ± MTX ± DMARD(s) (n = 1870)	All TCZ Doses (n = 2928)	Control* (n = 1271)
No. of patients providing samples for the screening assay	1028	1788	2816	702
Patients providing a sample that tested positive on the screening/confirmation assays at any time (including baseline), no. (%)	23 (2.2)	41 (2.3)	64 (2.3)	23 (3.3)
Patients providing samples that tested positive on the screening/confirmation assays at baseline, no. (%)	15 (1.5)	33 (1.8)	48 (1.7)	17 (2.4)

MTX = methotrexate; DMARD = disease-modifying antirheumatic drug.

*Placebo ± MTX ± DMARD(s).

syndrome), although one was subsequently classified as a hypersensitivity reaction. Another 6 patients (0.1%) had an infusion-related/hypersensitivity reaction. Other events that led to discontinuation of TCZ in 6 patients included combinations of various events, such as dyspnea, nasopharyngitis, pain, syncope, oral infection, influenza, dizziness, blister, pyrexia, headache, depression, meningioma, pneumonia, furuncle, and urinary tract infection.

Table III summarizes the results of AE-driven immunogenicity testing for the following 3 clusters of potentially immunogenic AEs that occurred during or within 24 hours after infusion, were considered related to TCZ therapy, and led to study withdrawal: anaphylactic reactions, infusion-related/hypersensitivity reactions, and other events. Among the 14 tested patients with an anaphylactic (n = 8) or infusion-related/hypersensitivity reaction (n = 6), 7 had previously tested positive on the screening and confirmation assays and 7 had tested negative. One of the patients with a negative result on the screening and confirmation assays tested positive on the subsequent SPR and IgE assays. In the remaining 6 patients, the negative result on the screening and confirmation assays was confirmed on the SPR and IgE assays. Among the 5 tested patients with other events (considered highly unlikely to have been caused by an immunogenic reaction), 4 had tested

negative on the screening and confirmation assays and 1 had tested positive. All 5 of these patients were negative on the SPR and IgE assays.

Of the 9 patients with an anaphylactic reaction (including the patient with a hypersensitivity reaction that was initially misclassified as an anaphylactic reaction), 5 tested positive for ADAs, 3 tested negative, and 1 was not available for testing. None of the patients with an anaphylactic reaction tested negative on the screening and confirmation assays and positive on the subsequent assays, indicating the adequacy of the screening ELISA to detect ADAs. However, the SPR and IgE assays were beneficial in correlating anaphylactic reactions with the presence of IgE ADAs, as was observed in 5 of 7 tested patients.

The results of the screening and confirmation assays, SPR assay, and IgE assay were highly consistent. Among the 18 patients for whom data were available on at least 2 of the 3 assays, 16 (88.9%) of the results were consistently positive or negative (**Table IV**).

Time Course of the Appearance of ADAs

Table V summarizes the time course of the appearance of ADAs in the 5 patients with anaphylactic reactions who tested positive on the assays. Three of the 5 patients tested ADA positive on all 3 assays before the onset of the reaction, and 1 tested positive on the

Table III. Results of immunogenicity testing in patients treated with tocilizumab who developed events leading to withdrawal from study medication. Data are number (%) of patients.

Variable	Anaphylactic Reaction (n = 9*)	Infusion-Related/ Hypersensitivity Reaction (n = 6)	Other Events (n = 6)	All Events (N = 21)
Patients providing samples for screening/ confirmation assays	8 (88.9)	6 (100)	5 (83.3)	19 (90.5)
Patients with samples that tested positive on screening/confirmation assays	5 (55.6)	2 (33.3)	1 (16.7)	8 (38.1)
Patients with samples that tested negative on screening/confirmation assay, but tested positive on SPR or IgE assay	0	1 (16.7)	0	1 (4.8)

SPR = surface plasmon resonance; IgE = immunoglobulin E.

*One patient was initially classified as having an anaphylactic reaction that was subsequently reclassified as a hypersensitivity reaction.

2 assays on which analysis was possible. The SPR assay did not detect ADAs at an earlier time point than did the screening and confirmation assays. The fifth patient was not available for testing at the time of the onset of the anaphylactic reaction but tested positive at the next available time point.

DISCUSSION

RA is a chronic disease, and special care must be taken to ensure that the benefits of drug treatment outweigh the risks. Detection of an unwanted immune response that may lead to untoward clinical sequelae is an important aspect of safety surveillance in clinical trials. Therefore, close analytic and clinical monitoring for immune reactions is necessary during drug treatment of patients with RA.

This study of TCZ applied an algorithm for immunogenicity testing in which clinical AEs played a crucial role. Use of the algorithm primarily served to assess the analytic validity of the ELISA in detecting ADAs, but it also provided clinical data for exploration of the potential relation between analytic and clinical immunogenicity data. Because the observation period in the clinical studies that provided data for the present analysis was limited to 24 weeks, occurrence of ADAs and clinical events of a potentially immunogenic nature on re-exposure to TCZ at later time points may not have been detected.

The typical immunogenicity testing strategy for biologic drugs employs a 2-stage process. In stage 1, dedicated blood samples are screened for the occurrence of ADAs. It is a regulatory requirement that this first step be designed to include a number of false-positive results⁵; therefore, an additional confirmation step is required to identify true positive results. In stage 2, only confirmed positive samples undergo further analysis to characterize the ADA response in terms of neutralizing potential, isotype, and/or recognized drug epitope. The neutralization potential of ADAs, which is not relevant to AEs, was not examined in the present study but is relevant to the assessment of efficacy.

The Phase III studies of TCZ included a second stage of analysis for AEs of a potentially immunogenic nature. A predefined set of AEs led to analysis of samples using the characterization assays, regardless of the result on stage-1 testing. Complementary ADA assays were used to minimize the risk of false-negative results, particularly in patients with a clinically relevant response (eg, anaphylactic reactions, hypersensitivity reactions, infusion-related reactions).

Based on data from 2816 patients who had received multiple doses of TCZ over a 24-week treatment period, the overall incidence of ADA formation associated with TCZ treatment was 2.3%. Based on data from 2315 patients who received TCZ in the extension studies, the incidence of ADA formation was 1.3%, similar

Table IV. Consistency of the results of immunogenicity assays of samples from patients treated with tocilizumab who had events of interest leading to withdrawal from study medication.

Type of Event	Result			Results Consistent?
	Screening/ Confirmation Assays	SPR Assay	IgE Immunoassay	
Anaphylactic reaction				
Patient A	Positive	Positive	Not tested	Yes
Patient B	Positive	Positive	Positive	Yes
Patient C	Positive	Positive	Positive	Yes
Patient D	Positive	Positive	Positive	Yes
Patient E	Positive	Positive	Positive	Yes
Patient F	Negative	Negative	Not tested	Yes
Patient G	Negative	Negative	Negative	Yes
Patient H	Not tested	Not tested	Not tested	NA
Patient I	Negative	Not tested	Not tested	NA
Infusion/hypersensitivity reaction				
Patient J	Negative	Positive	Positive	No
Patient K	Negative	Negative	Negative	Yes
Patient L	Positive	Positive	Positive	Yes
Patient M	Negative	Negative	Not tested	Yes
Patient N	Positive	Positive	Positive	Yes
Patient O	Negative	Negative	Negative	Yes
Other events				
Patient P	Negative	Negative	Not tested	Yes
Patient Q	Positive	Negative	Negative	No
Patient R	Negative	Negative	Negative	Yes
Patient S	Negative	Negative	Negative	Yes
Patient T	Negative	Negative	Negative	Yes
Patient U	Not tested	Not tested	Not tested	NA

SPR = surface plasmon resonance; IgE = immunoglobulin E; NA = not applicable.

to that after 24 weeks. Specific ADAs against various therapeutic agents have been detected at baseline, which may be explained by the cross-reaction of preexisting antibodies with one or more epitopes of the biologic agent, thereby precipitating anaphylactic-like reactions.^{11,24}

The stage-2 testing strategy included isotype analysis of ADAs, which was performed using 2 different methods. The SPR assay was used to detect all Ig subtypes,²¹ and the IgE assay,^{11,20} which has widely been used for the analysis of IgE antibodies in human samples, was adapted for specific analysis of IgE anti-TCZ antibodies. ADAs of all isotypes were found, either alone or in combination. No apparent correlation with clinical

observations emerged from the analysis of IgG and IgM, and no alterations in treatment were made as a result of findings on the isotype analysis. However, the isotyping assay was helpful in characterizing samples that were positive at baseline; this was found to be caused by an IgM response directed against the Fc of TCZ. A possible explanation for this response is the higher levels of rheumatoid factor in patients with RA.¹⁹ Eight of 14 patients (57.1%) with an anaphylactic reaction or infusion-related/hypersensitivity reaction tested positive for IgE. This compares well with values reported in small studies of infliximab (3/11)¹⁰ and cetuximab (17/25).¹¹ During method development, the SPR assay

Table V. Time course of the results of immunogenicity testing in patients treated with tocilizumab (TCZ) who tested positive for antidrug antibodies on the screening/confirmation assays before dosing and subsequently developed anaphylactic reactions.

Variable	Patient A	Patient B	Patient C	Patient D	Patient E
TCZ dose, mg/kg	4	8	4	4	4
Type of anaphylactic event	Anaphylactic reaction	Anaphylactic shock	Anaphylactic reaction	Anaphylactic shock	Anaphylactic reaction
Onset of event, by study week	8	4	8	4	8
Assay results					
Baseline					
Screening/confirmation assays	Negative	Negative	Negative	Negative	Negative
SPR assay	Negative	Negative	Negative	Positive	Positive
IgE assay	Not tested	Not tested	Not tested	Not tested	Not tested
Week 2					
Screening/confirmation assays	Not tested	Negative	Not tested	Not tested	Negative
SPR assay	Not tested	Not tested	Not tested	Not tested	Negative
IgE assay	Not tested	Positive	Not tested	Not tested	BLQ
Week 4					
Screening/confirmation assays	Not tested	Positive	Negative	Not tested	Negative
SPR assay	Not tested	Positive	Negative	Not tested	Negative
IgE assay	Not tested	Positive	BLQ	Not tested	BLQ
Week 8					
Screening/confirmation assays	Positive*	Positive*	Positive	Positive*	Positive
SPR assay	Positive	Positive	Positive	Positive	Positive
IgE assay	Not analyzed†	BLQ	Positive	Positive	Positive
Week 12					
Screening/confirmation assays	Not tested	Not tested	Positive*	Not tested	Not tested*
SPR assay	Not tested	Not tested	Positive	Not tested	Not tested
IgE assay	Not tested	Not tested	Not analyzed†	Not tested	Not tested

SPR = surface plasmon resonance; IgE = immunoglobulin E; BLQ = below limit of quantitation.

* Study medication was discontinued.

† Quantity of sample not adequate for analysis.

produced a number of false-negative results for ADAs of the IgE subtype, warranting use of an additional IgE assay.¹⁹

Only 21 of all treated patients in the Phase III clinical trials of TCZ had SAEs that led to discontinuation of study medication. When samples from these patients were subjected to clinical AE-driven immunogenicity testing, 6 of the events were considered unlikely to be mediated by immunologic mechanisms (eg, pneumonia, dizziness, influenza). Consistent with this assessment, only one patient tested positive on the ADA screening and confirmation assays and negative on the second-stage assays, which could have been an effect of the lower sensitivity of the SPR assay. Four of the 6 patients tested negative on all assays, and the other patient was not available for ADA testing.

In contrast, among the 14 tested patients with an AE in which an immunologic reaction was plausible given the clinical manifestation (eg, anaphylactic reaction, infusion-related/hypersensitivity reaction), 7 patients (50.0%) had a positive result on the ADA screening and confirmation assays and 7 had a negative result. One of the patients with a negative result on screening tested positive on the second-stage tests; the screening result was corrected to a false-negative and the patient was categorized as ADA positive. The stage-1 and stage-2 assays were conclusive in 88.9% of patients undergoing clinical AE-driven testing. In the 6 patients who tested negative for ADAs despite having had a hypersensitivity reaction, a mechanism independent of TCZ-specific ADAs may have been responsible; for example, acute immune toxicities related to complement activation,²⁵ idiosyncratic host-dependent drug hypersensitivity reactions to NSAIDs,²⁶ or nonimmune-type anaphylaxis from local anesthetics.²⁷ Because 3 complementary ADA assays were used, it is less likely that the ADA-negative results in the patients with infusion-related reactions were false-negatives. The occurrence of infusion reactions in the control group also suggested an ADA-independent mechanism.

In summary, the ELISA screening and confirmation assays generated only one false-negative result in this patient population. The ELISA has the advantage of high throughput, ease of handling, and cost-effectiveness in the timely testing of all Ig isotypes in a large number of samples. The SPR assay, which was used in second-stage testing, revealed a single false-negative result; however, it appeared to be less sensitive than the screening and confirmation ELISA, as it failed to detect ADAs

in a sample that had previously tested positive. These findings are consistent with previous observations that no single assay can meet all expectations,⁵ and a combination of complementary assays can be useful.

CONCLUSIONS

Based on the findings of this analysis in a relevant patient population, the bridging-type screening and confirmation ELISA was a valid method of detecting anti-TCZ ADAs. Immunogenicity testing of samples from patients with clinical AEs of a potentially immunogenic nature using assays complementary to the ELISA added valuable information about the incidence and character of ADAs. However, the lack of a gold standard limited the ability to correlate the incidence of ADAs with clinical events of an immunogenic nature. The application of clinical AE-driven immunogenicity testing to improve ADA assessment warrants further study.

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