Research paper

Reporter gene assay for the quantification of the activity and neutralizing antibody response to TNFα antagonists

Christophe Lallemand [a], Nadia Kavrochorianou [a,1], Casper Steenholdt [b], Klaus Bendtzen [c],
Mark A. Ainsworth [b], Jean-Francois Meritet [d], Brigitte Blanchard [a], Pierre Lebon [d], Peter Taylor [e],
Peter Charles [e], Saba Alzabin [e], Michael G. Tovey [a,*]

[a] Laboratory of Biotechnology and Applied Pharmacology, CNRS UMR8113, Ecole Normale Supérieure de Cachan, Cachan, France
[b] Department of Medical Gastroenterology, Herlev Hospital, Herlev, Denmark
[c] Institute for Inflammation Research, Rigshospitalet University Hospital, Copenhagen, Denmark
[d] Department of Virology, Cochin Hospital, University René Descartes, Paris, France
[e] Kennedy Institute of Rheumatology, Imperial College, London, UK

ABSTRACT

A cell-based assay has been developed for the quantification of the activity of TNFα antagonists based on human erythroleukemic K562 cells transfected with a NFκB regulated firefly luciferase reporter-gene construct. Both drug activity and anti-drug neutralizing antibodies can be quantified with a high degree of precision within 2 h, and without interference from cytokines and other factors known to activate NFκB. The assay cells also contain the Renilla luciferase reporter gene under the control of a constitutive promoter that allows TNFα-induced firefly luciferase activity to be normalized relative to Renilla luciferase expression. Thus, results are independent of cell number or differences in cell viability, resulting in intra and inter assay coefficients of variation of 10% or less. Normalization of results relative to the expression of an internal standard also provides a means for correcting for serum matrix effects and allows residual drug levels or anti-drug neutralizing antibodies to be quantified even in serum samples with a relatively high degree of cytotoxicity.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Antagonists of tumor necrosis alpha (TNFα) are used widely for the treatment of a number of chronic inflammatory or autoimmune diseases such as rheumatoid arthritis (RA), psoriasis, and Crohn's Disease (Lee and Fedorak, 2010; Scott et al., 2010; Furst et al., 2011). Such antagonists include; infliximab (Remicade®), a chimeric monoclonal antibody against TNFα, adalimumab (Humira®) and golimumab (Simponi®) fully human monoclonal anti-TNFα antibodies, etanercept (Enbrel®) a fusion protein comprising the p75 chain of the TNFα receptor and the Fc moiety of human IgG1, and certolizumab (Cimzia®) pegylated Fab fragments of a humanized anti-TNFα monoclonal antibody (Lee and Fedorak, 2010; Scott et al., 2010; Furst et al., 2011). Although TNFα antagonists are for the most part well tolerated their safety and efficacy can be compromised, however, by the development of an immune response against the therapeutic protein (Baert et al., 2003; Wolbink et al., 2006; Bendtzen et al., 2006, 2009; Aikawa et al., 2010). It is widely accepted that injection of foreign proteins into humans can elicit an immune reaction leading to the production of antibodies, it is also clear, however, that repeated injection of fully human proteins can result in a break in immune tolerance to self-antigens leading to a humoral response against the protein in some patients (Wadhwa and Thorpe, 2010). Although in many cases the immune response to a therapeutic protein has little or no clinical impact, anti-drug antibodies (ADAs) do,

* Corresponding author.
  E-mail address: michael.tovey@ens-cachan.fr (M.G. Tovey).
  [1] Present address: Laboratory of Transgenic Technology, Hellenic Pasteur Institute, Athens, Greece.

however, pose a number of potential risks for the patient particularly in the case of a neutralizing antibody (NAb) response. An ADA response can also adversely affect the safety of treatment and cause immune complex disease, and allergic reactions (Han and Cohen, 2004). Some cases of severe autoimmune reactions have also been reported where ADAs cross-react with a non-redundant endogenous counterpart (Casadevall et al., 2002).

Monitoring patients for the presence of ADAs capable of neutralizing drug activity is therefore an important part of drug safety evaluation since currently available techniques do not permit one to predict with a sufficient degree of accuracy whether a product will be immunogenic in a particular patient, and at what point during treatment an immune response will occur (Buttel et al., 2011). Regulatory authorities often recommend that cell-based assays be used to quantify anti-drug neutralizing antibodies when an appropriate assay is available (Kirshner, 2009; Thorpe, 2011). Cell-based assays for NAbs against TNFα antagonists are for the most part based on the ability of TNFα to induce cytotoxicity in susceptible cell lines such as actinomycin D treated mouse L929 cells or mouse WEHI cells (Ruff and Gifford, 1981; Bersani et al., 1986; Lucas et al., 1990; Ebert et al., 2008; Mann et al., 2008). Such assays are difficult to standardize, take 1 to 2 days to complete, and are subject to serum matrix effects (Ruff and Gifford, 1981; Bersani et al., 1986; Lucas et al., 1990; Meager, 2006; Gupta et al., 2007; Ebert et al., 2008; Mann et al., 2008). These limitations have been overcome by the development of a reporter-gene assay that allows both the activity and anti-drug neutralizing antibody response to TNFα antagonists to be quantified with a high degree of precision within 2 h, independent of serum matrix effects.

2. Materials and methods

2.1. Reporter cells

Human erythroleukemic K562 cells were transfected with a NFκB regulated reporter-gene construct. Briefly, a synthetic double-stranded oligonucleotide corresponding to a five fold tandem repeat of the NFkB recognition sequence (GGGGACTTTCCGCT), controlling a SV40 minimal promoter was cloned upstream of the firefly luciferase (FL) reporter-gene by insertion into the HindIII/XbaI site of the pGL2 vector (Promega, Madison, WI) and stable transfectants were isolated and cloned. A stable cell line, KK-Luc, carrying the FL reporter gene under the control of NFκB responsive chimeric promoter was thus established. These cells were then transfected with the coding sequence of the Renilla luciferase (RL) gene under the control of a constitutive minimal thymidine kinase promoter (Promega, Madison, WI). The integrity of each construct was verified by sequencing. Stable clones were isolated and tested for TNFα responsiveness. One such clone, KLJ-Luc was then characterized further. Master and working cell banks of KLJ-Luc cells were maintained in liquid nitrogen and vials were thawed prior to use and maintained in cell culture in the presence of 150 μg/ml of hygromycin B and 100 μg/ml of zeocine for at least 50 passages without loss of performance.

2.2. Quantification of TNFα activity

Increasing concentrations (0.001 to 1000 ng/ml) of human TNFα (R & D Systems, Minneapolis, MN) were prepared in 50 μl of RPMI 1640 medium + 10% FBS in a white opaque 96-well microtiter plate (Perkin Elmer, Waltham MA) and tested in duplicate. KJL-Luc cells (10,000 cell/well) were added in a total volume of 50 μl of RPMI 1640 medium + 10% FBS and incubated for 2 h at 37 °C in a CO_2 incubator. Dual-Glo (Promega, Medison WI) FL reagent was added (50 μl/well) and a first reading taken in a luminometer (TriStar LB 941, Berthold, Bad Wildbad, Germany). The Dual-Glo RL reagent (50 μl/well) was then added and a second reading taken. The readings were integrated into an Excel spread-sheet and FL activity in relative luciferase units (RLU) or FL activity normalized relative to constitutive RL activity were plotted against TNFα concentration.

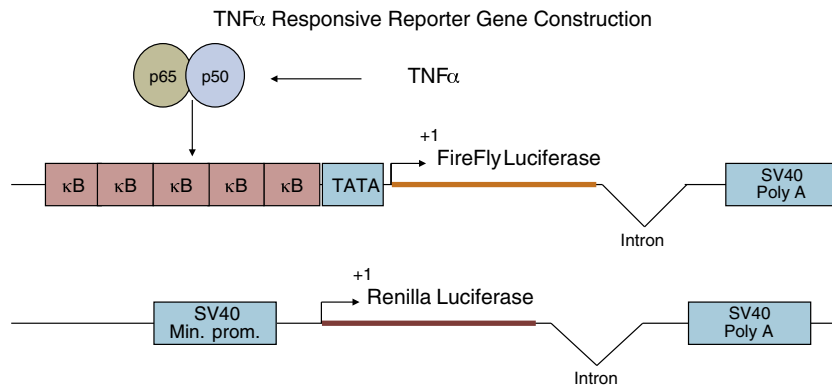### 2.3. Quantification of the activity of TNFα antagonists

Increasing concentrations (5 ng/ml to 500 ng/ml) of the TNFα antagonist (infliximab, adalimumab, or etanercept) were prepared in 50 μl of RPMI 1640 medium + 10% FBS in a white opaque 96-well microtiter plate (Perkin Elmer, Waltham MA) and then titrated in duplicate against a fixed concentration of human TNFα (2.0 ng/ml). The TNFα, antagonists were incubated with TNFα for 30 min at 37 °C prior to the addition of KJL-Luc cells. Residual TNFα activity was then quantified by interpolation of a standard curve of FL/RL activity in a simultaneous titration of TNFα activity as described above.

2.4. Quantification of neutralizing antibodies against TNFα antagonists

Serial four fold dilutions (1/4 to 1/16,000) of patient sera were prepared in a final volume of 50 μl of RPMI 1640 medium + 10% FBS in a white opaque 96-well microtiter plate (Perkin Elmer, Waltham MA) and tested in duplicate. Between two and three laboratory units:ml (LU/ml) of the TNFα antagonist used to treat the patient was then added to each well and incubated for 10 min at 37 °C. Two LU/ml (2.0 ng/ml) of human of TNFα, was then added and incubated for a further 30 min at 37 °C prior to the addition of KJL-Luc cells and the quantification of residual TNFα activity as described above.

Neutralizing titer was determined from the ratio of the FL/RL readings for a particular serum sample using the Kawade methodology which determines the reciprocal of the antibody dilution that reduces TNFα antagonist activity from 10 to 1.0 LU/ml (EC_{50}) according to the formula; $t = f(n-1)/9$, where f = the reciprocal of the antibody dilution, and n = TNFα antagonist concentration in LU/ml. Neutralization titers were corrected for the actual number (between 2 and 3) of LU/ml of the TNFα antagonist used in the neutralization assay from the value obtained in the simultaneous titration of TNFα activity, and are expressed as Ten Fold Reduction Units/ml or TRU/ml (Grossberg et al., 2001a,b, 2009; Kawade et al., 2003; Lallemand et al., 2008).

TNFα Responsive Reporter Gene Construction

Fig. 1. TNFα responsive reporter gene construction. κB: NFκB recognition sequence. TATA: TATA box. Firefly Luciferase: Coding region of the Firefly luciferase gene. Intron: Intron from the human β-globulin gene. SV40 Poly A: SV40 polyadenylation site. SV40 Min. Prom: SV40 minimal promoter. Renilla Luciferase: Coding region of the Renilla luciferase gene.

2.5. Patient sera

Archival samples of serum from patients with rheumatoid arthritis (RA) treated with infliximab, adalimumab, or etanercept, or from patients with Crohn's disease treated with infliximab, and monitored for the presence of anti-drug neutralizing antibodies, were randomly selected for evaluation in the present study.
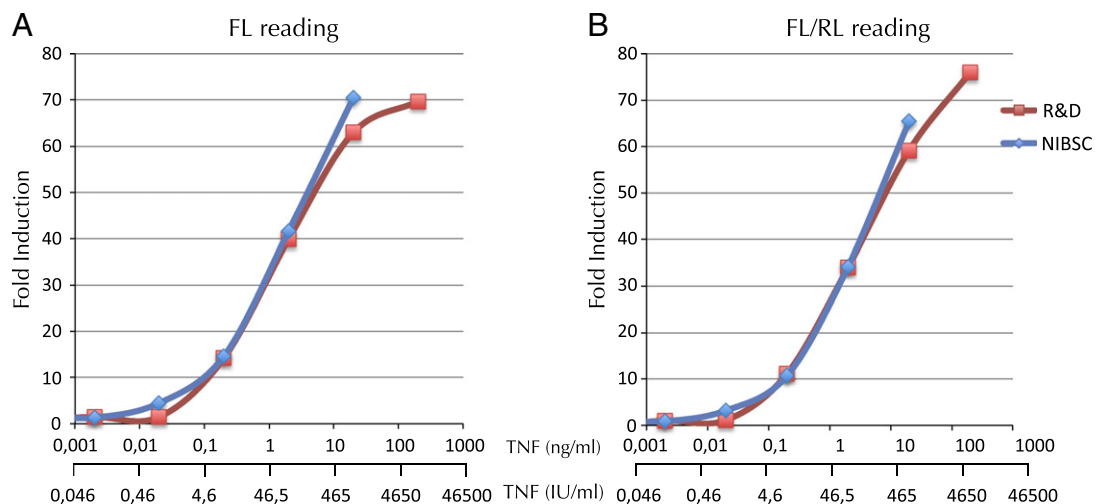
3. Results

3.1. Establishment of a TNFα responsive reporter gene cell line

Human erythroleukemic K562 cells were transfected with a NFκB regulated Firefly luciferase (FL) reporter-gene construct together with the Renilla luciferase (RL) reporter gene under the control of a constitutive promoter (Fig. 1) as described in the Materials and methods. Clones of stable transfectants were isolated and tested sequentially for both FL induction in response to treatment with TNFα and constitutive RL expression. A stable cell line, KJL-Luc was thus established and characterized further.

3.2. Response of KJL-Luc cells to treatment with TNFα

The response of KJL-Luc cells to TNFα was determined by treatment of reporter cells with varying concentrations of a standard preparation of human TNFα, or the WHO International TNFα Reference Reagent (NIBSC 88/786), for 2 h at 37 °C prior to addition of lysis buffer and determination of FL activity as described in the Materials and methods. A typical sigmoid dose response was obtained (Fig. 2A) with a standard error of $+/-5\%$ over a wide range of TNFα concentrations (0.01 to 100 ng/ml). As shown in Fig. 2 one laboratory unit (1.0 LU/ml) of the laboratory standard was equivalent to approximately 1 ng/ml or 46.5 international units/ml (IU/ml) and the lower limit of quantification was 0.01 ng/ml or approximately 0.5 IU/ml (Fig. 2A). A

Fig. 2. Response of KJL-Luc cells to treatment with TNFα. Duplicate samples of human TNFα (R & D Systems, Minneapolis, MN) or the WHO International TNFα Reference Reagent (NIBSC 88/786), at the concentrations indicated in the figure, were incubated with KJL-Luc reporter cells or 2 h at 37 °C prior to addition of the Dual Glo FL reagent (Promga, Medison WI) and determination of FL activity (Panel A) and the subsequent addition of the Dual Glo RL reagent (Promga, Medison WI) and the sequential determination of FL and RL activities (Panel B) as described in the Materials and methods.

similar dose response curve was observed when TNFα induced FL expression was normalized with respect to RL expression (Fig. 2B).

The level of TNFα induced FL activity, expressed as relative luciferase units (RLU), was found to increase with increasing cell number as shown in Fig. 3A. Normalization of TNFα induced FL expression relative to constitutive expression of RL activity renders results independent of cell number as shown in Fig. 3B.
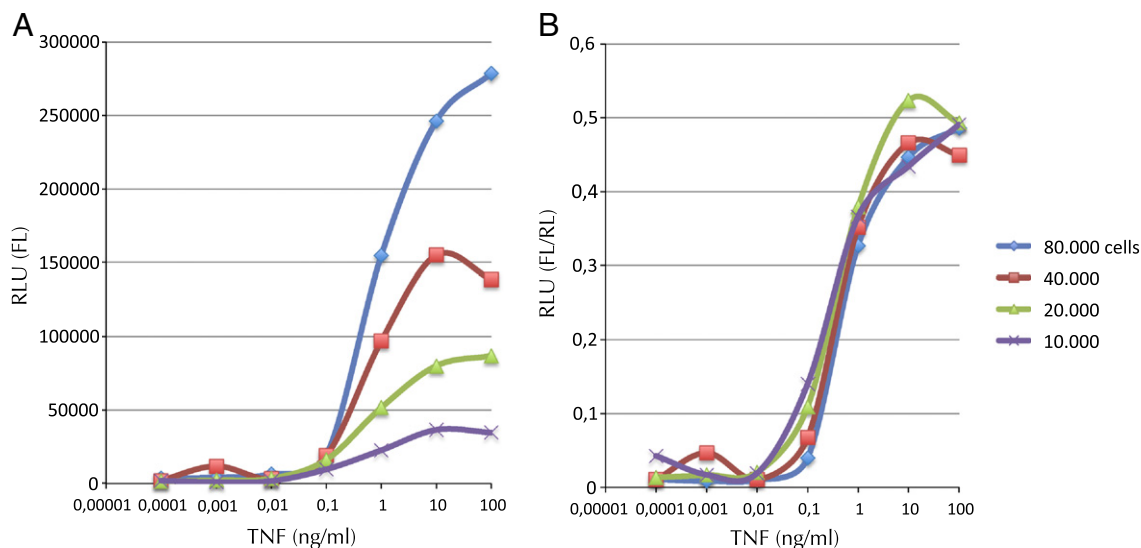
3.3. Specificity of the response of KJL-Luc cells to treatment with TNFα

Numerous cytokines and other factors in addition to TNFα can activate the NFκB pathway. Thus, a number of such factors were tested for their ability to activate the NFκB responsive FL reporter gene and hence potentially interfere with the assay (Fig. 4). None of the factors tested including IL-1β, TGF-β, and LPS was found to activate the reporter gene over the concentration range of the assay (0.01 to 100 ng/ml) either under standard assay conditions (2 h incubation) or even after incubation overnight (Fig. 4). Very high concentrations of LPS (5 to 10 μg/ml) were able, however, to activate the reporter gene following incubation overnight (data not shown).
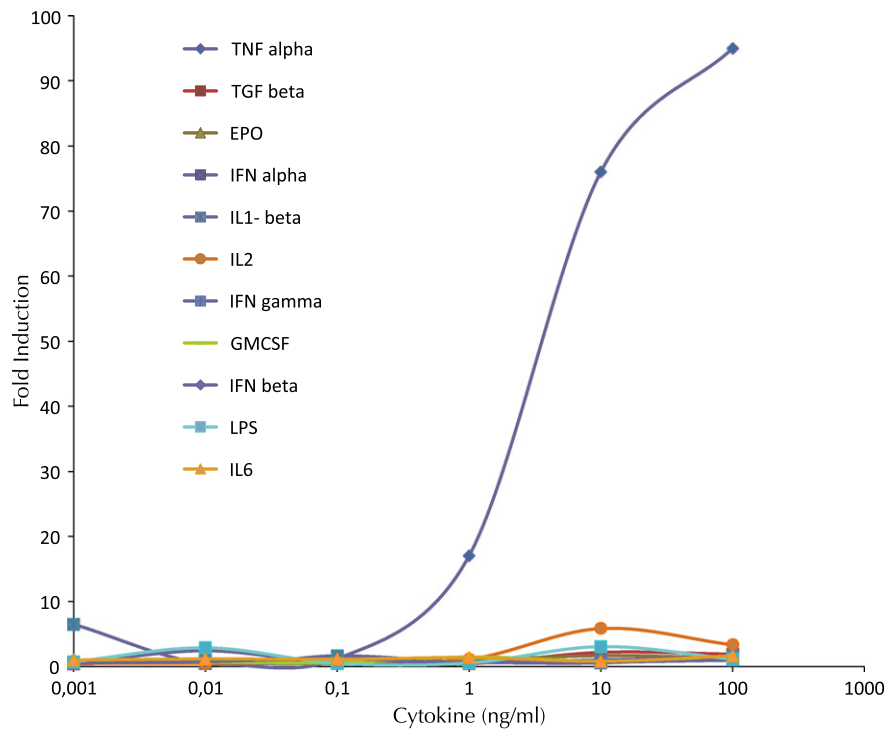
3.4. Response of KJL-Luc cells to serum matrix effects

Cell-based assays are often subject to serum matrix effects that can adversely affect the ability to detect drug activity or an ADA response in the presence of low dilutions of serum, in particular in serum from patients with autoimmune or inflammatory disease. To determine the effect of patient serum on TNFα induced FL expression, KJL-Luc cells were incubated for 18 h at 37 °C with increasing concentrations of TNFα in the presence of a 1/10 or 1/20 final dilution of a pool of sera from

patients with rheumatoid arthritis in RPMI 1640 medium with 10% FBS, or with 1/10 dilution of a pool of normal human serum, or with medium alone (Fig. 5A). A marked inhibition of TNFα induced FL expression was observed in the presence of serum from patients with rheumatoid arthritis over the whole range of TNFα concentrations tested, relative to the level of TNFα induced FL expression observed in the presence of either normal human serum, or medium alone (Fig. 5A). Normalization of TNFα induced FL expression relative to the constitutive expression of RL activity allowed results to be corrected for non-specific serum matrix effects (Fig. 5B). To determine the frequency and extent of serum matrix effects, individual samples of serum from patients with Crohn's disease were spiked with 2.0 LU of TNFα and incubated at a 1/10 final dilution with KJL-Luc cells for 2.0 h at 37 °C. FL expression was determined and the results compared to those obtained for samples containing the same dilution of a pool of normal human serum or to a control sample without human serum. As shown in Fig. 6A only 50 to 80% of the activity of added TNFα was detected in a number of the samples when compared to samples containing the same dilution of normal human serum, or a control sample without human serum. In contrast, full activity was obtained for the same samples following normalization of TNFα induced FL expression relative to RL expression, regulated by a constitutive promoter (Fig. 6A). Normalization of TNFα induced FL expression relative to RL expression, for samples for which only 20% or less of the activity of added TNFα could be recovered, also reduced serum matrix effects even though the recovery of TNFα activity remained low most probably reflecting the presence of residual infliximab in these samples (Fig. 6A). Similar results were obtained following normalization of TNFα induced FL expression relative to RL expression for samples from patients with RA treated with adalimumab, infliximab, or etanercept (data not shown). For some of the samples from patients with
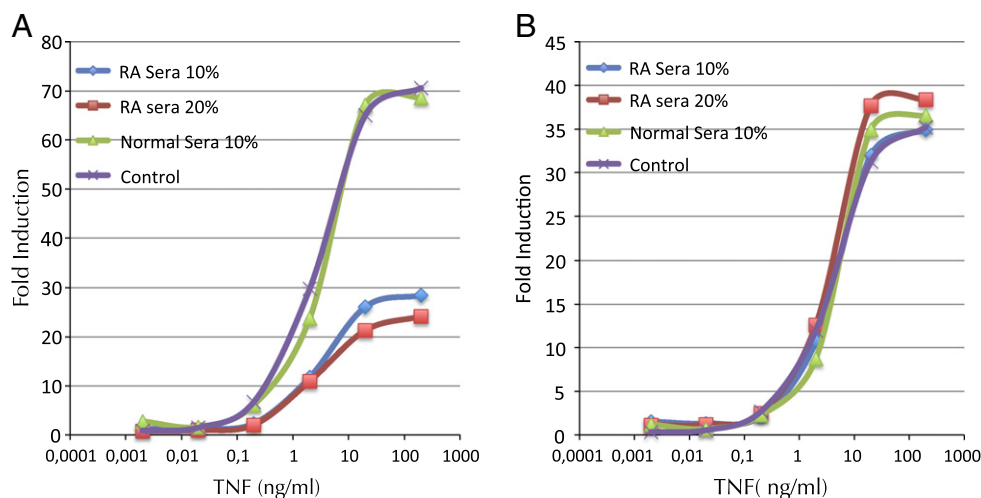

Fig. 3. Relationship between TNFα induced FL expression and cell number. Duplicate samples of human TNFα (R & D Systems, Minneapolis, MN) at the concentrations indicated in the figure, were incubated with the number of KJL-Luc reporter cells indicated in the figure for 2 h at 37 °C prior to addition of DualGlo (Promga, Medison WI) and the sequential determination of FL and RL activities as described in the Materials and methods. Panel A: TNFα induced FL activity. Panel B: TNFα induced FL activity normalized relative to constitutive expression of RL activity.

Fig. 4. Specificity of the response of KJL-Luc cells to treatment with TNFα. Duplicate samples of either human TNFα (R & D Systems, Minneapolis, MN) or TGF-β, EPO, IFNα, IFNβ, IFNγ, IL-1β, IL-2, IL-6, GM-CSF, or LPS at the concentrations indicated in the figure were incubated with KJL-Luc reporter cells for 18 h at 37 °C prior to addition of DualGlo (Promga, Medison WI) and the sequential determination of FL and RL activities as described in the Materials and methods. Results are expressed as fold induction relative to the control sample containing RPMI 1640 medium with 10% FBS alone.

Crohn's disease more than 100% of the activity of the TNFα added could be recovered following normalization of TNFα induced FL expression relative to RL expression (Fig. 6A). Further investigation revealed that the activity detected in these samples, in the absence of added TNFα was markedly reduced or abrogated following addition of 250 ng/ml of infliximab suggesting that this activity reflected the presence of endogenous TNFα in these samples (Fig. 7A). The presence of low levels of

Fig. 5. Response of KJL-Luc cells to serum matrix effects. Duplicate samples of human TNFα (R & D Systems, Minneapolis, MN) at the concentrations indicated in the figure, were incubated with KJL-Luc reporter cells in the presence of a 1/5 (20%), or 1/10 (10%) final dilution of a pool of serum from patients with rheumatoid arthritis, or a pool of normal human donor serum (Invitrogen, Carlsbad, CA), or medium alone for 18 h at 37 °C prior to addition of DualGlo (Promga, Medison WI) and the sequential determination of FL and RL activities as described in the Materials and methods. Panel A: TNFα induced FL activity. Panel B: TNFα induced FL activity normalized relative to constitutive expression of RL activity.

TNFα (5 to 50 pg/ml) in these samples was confirmed using an ELISA for human TNFα (QuantiGlo, R & D Systems, Minneapolis, MN) as shown in Fig. 7B.

3.5. Detection of circulating drug activity in serum from patients with Crohn's disease treated with infliximab

KJL-Luc cells provide the basis for a sensitive screening assay for the detection of circulating drug activity in patients with autoimmune or inflammatory diseases treated with TNFα antagonists. Thus, addition of 2 to 3 LU of TNFα to samples of serum from patients with Crohn's disease followed by normalization of TNFα induced FL expression relative to RL expression showed that the majority of samples segregated into two groups. Those for which 80 to 100% or more of the activity of added TNFα could be recovered, indicating the absence of circulating levels of active drug and most probably the presence of neutralizing anti-drug antibodies (Fig. 6A), and those for which 20% or less of the added TNFα activity could be recovered, indicating the presence of residual drug activity (Fig. 6A). Only 9 of 124 samples analyzed yielded intermediate results (Fig. 6A).

3.6. Quantification of the activity of TNFα antagonists

KJL-Luc cells can also be used to quantify the activity of different types of TNFα antagonists. Thus, increasing concentrations of the chimeric anti-TNFα MAb infliximab, the fully human MAb, adalimumab, and the TNFα receptor fusion protein etanercept were titrated against a fixed concentration of TNFα (2.0 ng/ml) in the presence of the KJL-Luc reporter cells as described in the Materials and methods (Fig. 8). In the case of infliximab, for example (Fig. 8), the mid-point of the dose response curve of % residual TNFα induced FL-activity normalized with respect to constitutive RL expression, was equivalent to a concentration of approximately 20 ng/ml or 1.0 LU/ml. The activity of TNFα antagonists present in sera from patients with RA treated with infliximab, adalimumab, or etanercept (Fig. 9A) and in sera from patients with Crohn's disease treated with infliximab (Fig. 6B) was readily quantified following normalization of FL activity relative to RL expression. Drug levels were determined from the activity of the dilution of the sample falling upon the linear portion of the dose–response curve of % residual TNFα activity against dilution in the presence of 2.0 ng/ml (2.0 LU/ml) of TNFα by interpolation of a

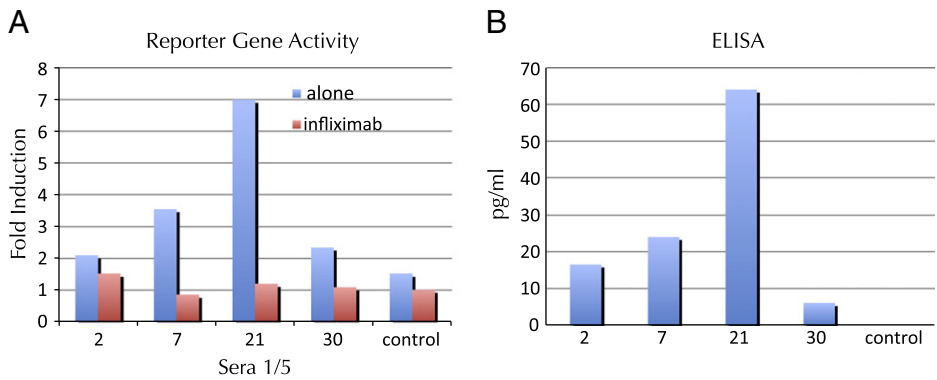standard curve of drug activity in a simultaneous titration also in the presence of 2.0 ng/ml of TNFα.

3.7. Quantification of neutralizing antibodies against TNFα antagonits in serum from patients with RA or Crohn's disease

Serial dilutions of serum from patients with RA treated with infliximab, adalimumab, or etanercept or serial dilutions of serum from patients with Crohn's disease treated with infliximab were mixed with 3.0 LU/ml the TNFα antagonist used to treat the patient, prior to the addition of 2.0 LU/ml of human of TNFα and incubation with KJL-Luc cells for 2 h at 37 °C. The Dual-Glo reagent (Promega, Madison, WI) was then added and FL and RL expressions were determined sequentially in the same sample as described in the Materials and methods. Neutralizing titer was determined from the ratio of the FL/RL readings for a particular serum sample using the Kawade methodology that determines the reciprocal of the antibody dilution that reduces TNFα antagonist activity from 10 to 1.0 LU/ml (EC_{50}). Results are expressed as TRU/ml (Grossberg et al., 2001a,b; Kawade et al., 2003; Grossberg et al., 2009). Thus, for each sample neutralizing titer was determined from the dilution that reduced the activity of 3.0 LU/ml (60 ng/ml) of infliximab to 1.0 LU/ml (20 ng/ml) in the presence of 2.0 LU/ml (2.0 ng/ml) of human TNFα. For example, for sample #2 (Fig. 6C) a 1/200 dilution of the sample was found to reduce the activity of 3.0 LU/ml of infliximab to 1.0 LU/ml which when corrected for the actual number of LU/ml of infliximab activity used in the neutralization assay from the value obtained in the simultaneous titration of infliximab activity according to the formula $t = 1/200 * (3−1)/9$, yielded a titer of 44 TRU/ml (Fig. 6C). The results obtained confirmed that those samples that appeared to contain neutralizing activity against TNFα antagonist in the screening assay (Fig. 6A), did indeed contain anti-drug neutralizing antibodies when titrated against a constant concentration of the TNFα antagonist (infliximab) used to treat the patient (Fig. 6C). Similarly, anti-drug NAbs could readily be detected in sera from patients with RA treated with infliximab, or adalimumab (Fig. 9B). No anti-entanercept NAbs were detected in the samples from patients with RA tested (Fig. 9B).

3.8. Determination of intra-assay and inter-assay coefficients of variation

Sera from patients with Crohn's disease treated with infliximab were tested for the presence of anti-infliximab NAbs as
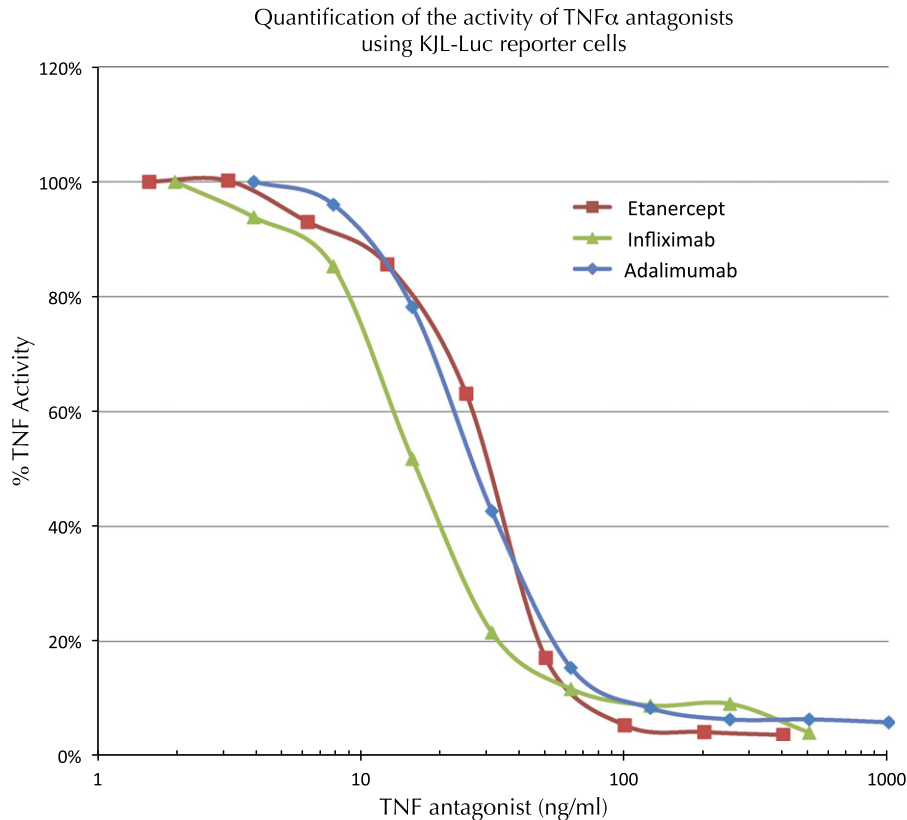
Fig. 6. A. Effect of sera from patients with Crohn's disease on the response of KJL-Luc cells to treatment with TNFα. Samples of serum from patients with Crohn's disease treated with infliximab were tested in duplicate at a 1/10 final dilution for their effect on the activation of KJL-Luc reporter cells in the presence of 2.0 ng/ml of human TNFα (R & D Systems, Minneapolis, MN). Following incubation for 2 h at 37 °C and addition of DualGlo (Promga, Medison WI), FL and RL activities were determined sequentially in the same sample as described in the Materials and methods. Results are expressed as a percentage of the activity of added TNFα relative to a control sample containing a 1/10 dilution of a pool of normal human serum (Invitrogen, Carlsbad, CA). **XXXXXX**: FL reading alone. **XXXXXX**: FL reading normalized with respect to RL expression. B. Quantification of infliximab activity in serum from patients with Crohn's disease. Serial ten fold dilutions (1/10 to 1/100,000) of samples of serum from patients with Crohn's disease treated with infliximab that exhibited <50% of FL/RL normalized TNFα activity depicted in Panel A were titrated in duplicate against a fixed concentration of TNFα (2.0 ng/ml) using KJL-Luc cells as described in the Materials and methods. Infliximab concentration was determined by interpolation of the curve of the simultaneous titration of FL/RL activity against infliximab concentration. Results are expressed as infliximab concentration (ng/ml). C. Quantification of anti-infliximab NAbs in sera from patients with Crohn's disease. Samples exhibiting >80% of FL/RL normalized TNFα activity depicted in Panel A were analyzed for the presence of anti-infliximab NAbs using KJL-Luc reporter cells in the presence of 3.0 LU/ml of infliximab as described in the Materials and methods. Neutralizing titer was determined from the ratio of the FL/RL readings for a particular serum sample using the Kawade methodology that determines the reciprocal of the antibody dilution that reduces TNFα antagonist activity from 10 to 1.0 LU/ml. Neutralization titers were corrected for the actual number of LU/ml of the TNFα antagonist used in the neutralization assay from the value obtained in the simultaneous titration. Results expressed as TRU/ml (Grossberg et al., 2001a,b, 2009; Kawade et al., 2003).

Fig. 7. Quantification of endogenous TNFα activity in sera from patients with Crohn's disease. Panel A. Samples of serum from patients with Crohn's disease treated with infliximab were tested in duplicate at a 1/5 final dilution for their effect on the activation of KJL-Luc reporter cells without the addition of exogenous TNFα either alone (*XXXXXX*), or in the presence of 50 ng/ml of infliximab (*XXXXXX*). Panel B. Samples of serum from patients with Crohn's disease treated with infliximab and tested in Panel A were analyzed in duplicate for the presence of TNFα using an ELISA specific for human TNFα (QuantiGlo, R & D Systems, Minneapolis, MN). Results are expressed as pg of TNFα/ml.
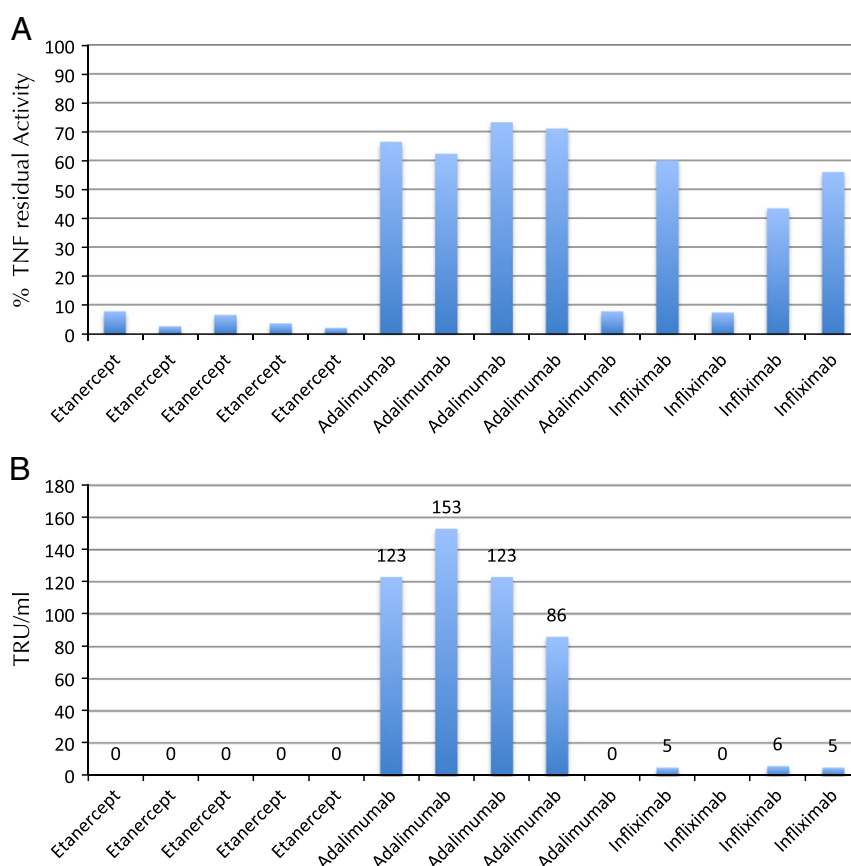
described in the Materials and methods. The intra-assay coefficient of variation was evaluated using four sera assayed four times on the same day by two different operators. The intra-assay coefficient of variation ranged from 2.1 to 7.6% when determined from FL readings normalized with respect to RL expression (Table 1). Inter-assay variability was evaluated using four sera assayed four times on each of four separate micro-titer plates following normalization of FL results relative to RL expression. The inter-assay coefficient of variation ranged from 6.6 to 9.4% (Table 1).

Fig. 8. Quantification of the activity of TNFα antagonists. Increasing concentrations of infliximab, adalimumab, or etanercept were titrated in duplicate against a fixed concentration of TNF α (2.0 ng/ml) using KJL-Luc cells as described in the Materials and methods. Results were expressed as% of the initial TNFα induced FL activity normalized with respect to constitutive expression of RL activity.

Fig. 9. Quantification of the activity of TNFα antagonists and anti-TNFα antagonist NAbs in sera from patients with RA. Panel A. Samples of serum from patients with RA treated with infliximab, adalimumab, or etanercept were tested in duplicate at a 1/10 final dilution for their effect on the activation of KJL-Luc reporter cells in the presence of 2.0 ng/ml of human TNFα (R & D Systems, Minneapolis, MN). Following incubation for 2 h at 37 °C and addition of DualGlo (Promega, Madison WI), FL and RL activities were determined sequentially in the same sample as described in the Materials and methods. Results are expressed as a percentage of the activity (FL/RL) of added TNFα relative to a control sample containing a 1/10 dilution of a pool of normal human serum (Invitrogen, Carlsbad, CA). Panel B. Serial four fold dilutions (1/4 to 1/16,384) of samples of serum from patients with RA treated with infliximab, adalimumab, or etanercept were titrated against a fixed concentration (3.0 LU/ml) of the TNF α antagonist used to treat the patient using KJL-Luc cells as described in the Materials and methods. Neutralizing titer was determined from the ratio of the FL/RL readings for a particular serum sample using the Kawade methodology and expressed as TRU/ml following correction for the actual number of LU/ml of the TNFα antagonist used in the neutralization assay employing the Kawade formula (Grossberg et al., 2001a,b, 2009; Kawade et al., 2003), and the value obtained in the simultaneous titration of drug activity as described in the Materials and methods.

3.9. Quantification of anti-infliximab neutralizing antibody levels using frozen cells

We have shown previously that cells transfected with a drug responsive reporter gene and treated with vinblastin can be stored frozen for extended periods without loss of drug responsiveness (Lallemand et al., 2008, 2010). In keeping with these results, vinblastin treated frozen assay-ready KJL-Luc cells were found to exhibit a similar degree of TNFα sensitivity as untreated cells, as reflected by the detection of similar levels of anti-infliximab NAbs in serum samples from patients with Crohn's disease (Table 2).

4. Discussion

The reporter-gene assay for TNFα described herein allows the activity and neutralizing antibody response to TNFα antagonists to be quantified with a high degree of precision within 2 h without interference from cytokines and other

factors known to activate NFκB. Thus, although the TNFα responsive reporter-gene construct used in this study is based upon a tandem repeat of a NFκB recognition sequence, cytokines such as IL-1β, TGF-β and IFNγ, and factors such as LPS

Table 1
Intra-assay and inter-assay variation of neutralizing anti-infliximab antibody titer of samples from patients with Crohn's disease.

Sample number	Nab TRU/ml	% CV intra-assay	% CV inter-assay
119	8.94	3.6	6.6
190	63.8	2.1	7.2
445	3.8	7.3	8.3
587	70.0	7.6	9.4

Intra-assay and inter-assay variability was evaluated using sera from patients with Crohn's disease treated with infliximab. Samples were assayed four times on the same day by two different operators or four times on each of four separate microtiter plates and the percentage coefficients of variations calculated.

Table 2
Neutralizing antibody titer of anti-drug antibodies from patients with Crohn's disease treated with infliximab.

Sample number	Antibody titer (TRU/ml)	
	Untreated cells	Vinblastin treated cells
418	400	600
438	3.8	7
580	70	250
617	15	25

Serial four fold dilutions (1/4 to 1/16,000) of each serum sample were prepared in 50 μl of RMPI 1640 medium + 10% FBS in a white opaque luminometer 96-well microtiter plate and tested in duplicate. Three LU/ml (60 ng/ml) of Infliximab was added to each sample and incubated for 10 min at 37 °C. Two LU/ml (2.0 ng/ml) of human TNFα was then added and the samples incubated for a further 10 min at 37 °C. Untreated, or freshly thawed vinblastin treated assay-ready frozen KJL-Luc cells were then added to each well in a total volume of 50 μl of RMPI 1640 medium + 10% FBS and incubated for 2 h at 37 °C in a CO_2 incubator. DualGlo (Promega, Madison, WI) added to each well and FL and RL activities were quantified in a luminometer as described in the Materials and methods. The dilution that reduced the activity of 3.0 LU/ml (60 ng/ml) of infliximab to 1.0 LU/ml (20 ng/ml) in the presence of 2.0 LU/ml (2.0 ng/ml) of human TNFα was determined for each sample. The results presented in the Table are the mean of two independent determinations.

that can activate NFκB, were found not to interfere with the assay at physiological levels over the time span and concentration range of the assay. Prolonged treatment (>18 h) of KJL-Luc reporter cells with a 100 fold higher concentration of LPS (10 μg/ml) did, however, activate the transgene (data not shown). Conventional cell-based assays for NAbs against TNFα antagonists are based on the ability of TNFα to induce cytotoxicity in susceptible cell lines such as actinomycin D treated mouse L929 cells or mouse WEHI cells (Ruff and Gifford, 1981; Bersani et al., 1986; Lucas et al., 1990; Ebert et al., 2008; Mann et al., 2008). Such assays take 1 to 2 days to complete, are difficult to standardize, and often give variable results (Meager, 2006; Gupta et al., 2007). In particular these assays are limited by their specificity since numerous factors present in serum from patients with inflammatory disease can affect cellular cytotoxicity and potentially interfere with the assay (Yucel et al., 2007). Furthermore, the sensitivity of cell-based assays is limited by the amount of human serum that cells can tolerate and hence the minimum sample dilution that can be employed (Yu et al., 2006; Wala et al., 2007). In this study marked serum matrix effects were observed with sera from both patients with RA and from patients with Crohn's disease as reflected by the inability to recover in full, the activity of TNFα added to the sample.

Normalization of drug-induced FL expression relative to RL expression, regulated by a constitutive promoter within the same cells, provides a means for correcting for the serum matrix effects observed when monitoring FL expression alone, most probably by compensating for non-specific effects on transcription and/or translation. Furthermore, since assay results are normalized relative to the expression of an internal standard, results are independent of cell number or differences in cell viability, resulting in inter- and intra-assay coefficients of variation of 10% or less. Most importantly, normalization of results relative to the expression of an internal standard allows residual drug levels or ADA

NAb levels to be determined even in serum samples with a relatively high degree of cytotoxicity (unpublished results).

The ability to correct results for serum matrix effects allows the KJL-Luc reporter cells to be used as the basis for a sensitive screening assay for the detection of circulating levels of drug activity in samples from patients with inflammatory disease treated with TNFα antagonists, simply by adding 2.0 ng/ml of TNFα to the samples followed by addition of the reporter cells. The precise levels of drug activity and neutralizing anti-drug antibodies present in a sample can then be quantified subsequently using the KJL-Luc reporter cells if required. Thus, active drug levels could readily be determined in sera from patients with RA treated with infliximab, adalimumab, or etanercept, and in patients with Crohn's disease treated with infliximab by interpolation of a standard curve of drug activity against concentration in a simultaneous titration.

Similarly anti-drug NAbs could be quantified in the sera of patients with RA treated with anfliximab or adalimumab or in sera from patients with Crohn's disease treated with infliximab. The Kawade methodology has been used extensively to quantify neutralizing antibodies against type I interferons and more recently NAbs against IL-6 (Grossberg et al., 2001a,b, 2009; Kawade et al., 2003). To our knowledge this is the first report of the use of the Kawade methodology to quantify Nabs against drugs that neutralize TNFα activity. In order to increase assay sensitivity, 3.0 LU/ml instead of 10 LU/ml of the drug was used in the neutralization assay, and corrected for the actual number of LU/ml of the TNFα antagonist employed from the value obtained in the simultaneous titration, using the Kawade formula. This simple adjustment markedly increased the sensitivity of ADA detection obtained from interpolation of two neutralization curves; TNFα activity by the TNFα antagonists, and TNFα antagonist activity by anti-drug NAbs. The failure to detect anti-etanercept NAbs in the samples tested from patients with RA is in keeping with the low incidence of anti-etanercept NAbs reported previously (Dore et al., 2007).

The reporter gene assay described herein is ideally suited for high throughput quantification of residual drug activity and anti-drug NAb levels in samples of serum from patients with inflammatory disease treated with TNFα antagonists. In addition, chemically treated cells can be manufactured in a cGMP facility and assay-ready cells stored at −80 °C for extended periods without loss of sensitivity or the need for cell culture (Lallemand et al., 2008, 2010). This assay is applicable to the quantification of the activity or NAb response to a variety of different TNFα antagonists including innovator products and biosimilars. Furthermore, the availability of a standardized assay format that can be used to assess the NAb response to a variety of different drugs will facilitate the comparison of immunogenicity data.

References

Aikawa, N.E., Sallum, A.M., Leal, M.M., Bonfa, E., Pereira, R.M., Silva, C.A., 2010. Menstrual and hormonal alterations in juvenile dermatomyositis. Clin. Exp. Rheumatol. 28, 571.

Baert, F., Noman, M., Vermeire, S., Van Assche, G., G.D.H., Carbonez, A., Rutgeerts, P., 2003. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N. Engl. J. Med. 348, 601.

Bendtzen, K., Geborek, P., Svenson, M., Larsson, L., Kapetanovic, M.C., Saxne, T., 2006. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. Arthritis Rheum. 54, 3782.

Bendtzen, K., Ainsworth, M., Steenholdt, C., Thomsen, O., Brynskov, J., 2009. Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. Scand. J. Gastroenterol. 44, 774.

Bersani, L., Colotta, F., Mantovani, A., 1986. Involvement of tumour necrosis factor in monocyte-mediated rapid killing of actinomycin D-pretreated WEHI 164 sarcoma cells. Immunology 59, 323.

Buttel, I.C., Chamberlain, P., Chowers, Y., Ehmann, F., Greinacher, A., Jefferis, R., Kramer, D., Kropshofer, H., Lloyd, P., Lubiniecki, A., Krause, R., Mire-Sluis, A., Platts-Mills, T., Ragheb, J.A., Reipert, B.M., Schellekens, H., Seitz, R., Stas, P., Subramanyam, M., Thorpe, R., Trouvin, J.H., Weise, M., Windisch, J., Schneider, C.K., 2011. Taking immunogenicity assessment of therapeutic proteins to the next level. Biologicals 39, 100.

Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P., 2002. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. N. Engl. J. Med. 346, 469.

Dore, R.K., Mathews, S., Schechtman, J., Surbeck, W., Mandel, D., Patel, A., Zhou, L., Peloso, P., 2007. The immunogenicity, safety, and efficacy of etanercept liquid administered once weekly in patients with rheumatoid arthritis. Clin. Exp. Rheumatol. 25, 40.

Ebert, E.C., Das, K.M., Mehta, V., Rezac, C., 2008. Non-response to infliximab may be due to innate neutralizing anti-tumour necrosis factor-alpha antibodies. Clin. Exp. Immunol. 154, 325.

Furst, D.E., Keystone, E.C., Braun, J., Breedveld, F.C., Burmester, G.R., De Benedetti, F., Dorner, T., Emery, P., Fleischmann, R., Gibofsky, A., Kalden, J.R., Kavanaugh, A., Kirkham, B., Mease, P., Sieper, J., Singer, N.G., Smolen, J.S., Van Riel, P.L., Weisman, M.H., Winthrop, K., 2011. Updated consensus statement on biological agents for the treatment of rheumatic diseases, 2010. Ann. Rheum. Dis. 70 (Suppl. 1), i2.

Grossberg, S.E., Kawade, Y., Kohase, M., Klein, J.P., 2001a. The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the tenfold reduction unit. J. Interferon Cytokine Res. 21, 743 (Page 20 01/09/2011).

Grossberg, S.E., Kawade, Y., Kohase, M., Yokoyama, H., Finter, N., 2001b. The neutralization of interferons by antibody. I. Quantitative and theoretical analyses of the neutralization reaction in different bioassay systems. J. Interferon Cytokine Res. 21, 729.

Grossberg, S.E., Casey, M., Grossberg, L.D., 2009. Quantification of the neutralization of cytokine biological activity by antibody: the ten-fold reduction bioassay of interleukin-6 as growth factor. J. Interferon Cytokine Res. 29, 421.

Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E., 2007. Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. J. Immunol. Methods 321, 1.

Han, P.D., Cohen, R.D., 2004. Managing immunogenic responses to infliximab: treatment implications for patients with Crohn's disease. Drugs 64, 1767.

Kawade, Y., Finter, N., Grossberg, S.E., 2003. Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affinity. J. Immunol. Methods 278, 127.

Kirshner, S., 2009. Guidance for industry assay development for immunogenicity testing of therapeutic proteins. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf.

Lallemand, C., Meritet, J.F., Erickson, R., Grossberg, S.E., Roullet, E., Lyon-Caen, O., Lebon, P., Tovey, M.G., 2008. Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. J. Interferon Cytokine Res. 28, 393.

Lallemand, C., Meritet, J.F., Blanchard, B., Lebon, P., Tovey, M.G., 2010. One-step assay for quantification of neutralizing antibodies to biopharmaceuticals. J. Immunol. Methods 356, 18.

Lee, T.W., Fedorak, R.N., 2010. Tumor necrosis factor-alpha monoclonal antibodies in the treatment of inflammatory bowel disease: clinical practice pharmacology. Gastroenterol. Clin. North Am. 39, 543.

Lucas, R., Heirwegh, K., Neirynck, A., Remels, L., Van Heuverswyn, H., De Baetselier, P., 1990. Generation and characterization of a neutralizing rat anti-rmTNF-alpha monoclonal antibody. Immunology 71, 218.

Mann, D.L., Bozkurt, B., Torre-Amione, G., Soran, O.Z., Sivasubramanian, N., 2008. Effect of the soluble TNF-antagonist etanercept on tumor necrosis factor bioactivity and stability. Clin. Transl. Sci. 1, 142.

Meager, A., 2006. Measurement of cytokines by bioassays: theory and application. Methods 38, 237.

Ruff, M.R., Gifford, G.E., 1981. Rabbit tumor necrosis factor: mechanism of action. Infect. Immun. 31, 380.

Scott, D.L., Wolfe, F., Huizinga, T.W., 2010. Rheumatoid arthritis. Lancet 376, 1094.

Thorpe, R., 2011. Biosimilar medicinal products. http://www.ema.europa.eu/docs/en_GB/document_library/Brochure/2011/03/WC500104228.pdf.

Wadhwa, M., Thorpe, R., 2010. Unwanted immunogenicity: lessons learned and future challenges. Bioanalysis 2, 1073.

Wala, I., Swanson, S.J., Jing, S., 2007. A non-radioactive method for detecting neutralizing antibodies against therapeutic proteins in serum. J. Pharm. Biomed. Anal. 45, 583.

Wolbink, G.J., Vis, M., Lems, W., Voskuyl, A.E., de Groot, E., Nurmohamed, M.T., Stapel, S., Tak, P.P., Aarden, L., Dijkmans, B., 2006. Development of antiinfliximab page 21 01/09/2011 antibodies and relationship to clinical response in patients with rheumatoid arthritis. Arthritis Rheum. 54, 711.

Yu, Y., Piddington, C., Fitzpatrick, D., Twomey, B., Xu, R., Swanson, S.J., Jing, S., 2006. A novel method for detecting neutralizing antibodies against therapeutic proteins by measuring gene expression. J. Immunol. Methods 316, 8.

Yucel, A., Aral, A.L., Basturk, B., Karakus, R., Aybay, C., 2007. Investigation of the functional characteristics of antibodies to therapeutic anti-human tumor necrosis factor alpha monoclonal antibody. Immunol. Lett. 111, 84.