Formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in cynomolgus monkeys.

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List of abbreviations:

AUC: area under the curve
CD: Crohn’s disease
CH50: total complement hemolytic activity
CL: clearance
Cmax: maximum plasma concentration
cpm: counts per minute
CR1: complement receptor-1
DPBS: Dulbecco’s phosphate buffered saline
EDTA: ethylene diamine tetra-acetic acid
ELISA: enzyme-linked immunosorbent assay
HPLC: high-performance liquid chromatography
IFX: infliximab
IgG: immunoglobulin G
RA: rheumatoid arthritis
RBC: red blood cell
RIA: radioimmunoassay
t½α: alpha half-life
t½β: beta half-life
TCA: trichloroacetic acid
TNFα: tumor necrosis factor alpha
Vz: volume of distribution
Recommended section assignment: Inflammation and Immunopharmacology
ABSTRACT

Infliximab (IFX) is a chimeric IgG1 monoclonal antibody specific for human tumor necrosis factor alpha (TNF) that is approved in the United States and Europe for the treatment of rheumatoid arthritis (RA) and Crohn’s disease (CD). Approximately 10% of RA and CD patients receiving maintenance treatment with IFX will develop antibodies to IFX. The objective of this study was to develop a model to assess the \textit{in vivo} formation, distribution, and elimination of immune complexes resulting from a low level immune response in the presence of excess concentration of a therapeutic antigen. In this model, cynomolgus monkeys were treated with a single intravenous injection of IFX, followed by injection of either radiolabeled, purified monkey anti-IFX immunoglobulin G (IgG) antibody (n=3, test group) or radiolabeled monkey non-immune IgG (n=3, control group). High-performance liquid chromatography analysis of collected sera revealed a rapid formation of immune complexes comprised of IFX – and radiolabeled anti-IFX IgG antibody immune complexes. The terminal half-life of the anti-IFX IgG antibody immune complex was approximately 38 hrs compared with 86 hrs for the non-immune antibody. However, the pharmacokinetic profile of IFX, although slightly lower in concentration over time for the test group, was not notably different relative to the control group. There were no macroscopic or microscopic histological findings in either treatment group. These data confirm that immune complexes between IFX and anti-IFX IgG antibodies can form \textit{in vivo} and that these immune complexes are eliminated more rapidly than non-immune antibodies in the presence of excess IFX.
It is generally accepted that all exogenous biological therapeutic proteins are capable of eliciting an immune response (Schellekens, 2002). Antibody responses to these therapeutic proteins can potentially lead to immune-mediated adverse events and/or reduced drug efficacy. Infliximab, a human/murine chimeric monoclonal IgG1κ antibody specific for human tumor necrosis factor alpha (TNFα), is approved for the chronic treatment of rheumatoid arthritis and Crohn’s disease. As with other approved biological therapies, some patients receiving infliximab can develop an immune response (Hanauer et al., 2004). The immune response can be characterized by the in vitro detection of circulating anti-therapeutic antibodies, however the commonly used analytical methods for antibody detection are subject to interference by long-lasting biotherapeutic drug present in the circulation. The coincident presence of both biologic drug and immune antibodies in vivo can result in immune complex formation, which can limit the detection of either the biologic drug or the antibody response. The analytical challenge of measuring an antibody response in the presence of soluble drug may further be confounded by an enhanced elimination of this higher molecular weight immune complex.

To date, only limited information is available on the formation and clearance of immune complexes consisting of a biologic agent and immune antibodies (Davies et. al., 1990). The immune complex clearance rate, consequences, and mechanism, along with the extent of tissue distribution, may be therapeutic agent-specific based on the size of the resulting immune complex. The size of the complex likely depends on a variety of factors including the size and structure of the therapeutic agent; the valency, concentration, and stoichiometry of the immune complex components; and whether the antibody response is directed to multiple epitopes on the biologic agent (Schifferli et. al., 1989).
Antibodies to infliximab are detected using validated enzyme-linked immunosorbent assay (ELISA) methodology (Centocor Standard Operating Procedure). The antibody status of patients without a positive immune response but with detectable concentrations of infliximab in their sera is conservatively categorized as inconclusive because binding of immune antibodies to the soluble biotherapeutic agent may compromise the detection of low-level immune responses. Currently, no immunogenicity assays are reported to be capable of detecting specific antibody responses in the presence of excess circulating therapeutic agent; although, some assay types may be more susceptible to this type of interference than others. Regardless, the relevance of an immune-response analysis to detect an antibody response in the presence of excess biotherapeutic agent is questionable if the immune complexes are rapidly eliminated in vivo after their formation (Johansson et al., 2002). Thus, if antibody-therapeutic biologic agent immune complexes are formed and rapidly eliminated in vivo, it may not be possible to detect, and it is probably not possible to accurately quantify, the immune antibody in presence of excess biologic agent (antigen).

This study was conducted to determine if infliximab-anti-infliximab immune complexes form in vivo and to assess the whole-body distribution and rate and mode of elimination of such complexes relative to non-immune antibodies.
METHODS

Experimental Design and Procedures

Naive, mauritius, female cynomolgus monkeys (*Macaca fascicularis*) were provided by Charles River Laboratories (Houston, TX). Monkeys were screened and determined to be negative for Herpes B virus, simian retrovirus and simian immunodeficiency virus. The animals were individually caged and quarantined for 10 days prior to study initiation in accordance with standards set forth in *The Guide for the Care and Use of Laboratory Animals* (1996).

Ten animals were screened for red blood cell (RBC) complement receptor-1 (CR1) receptor density, using a quantitative CR1 receptor assay, prior to study initiation. Six animals were identified for use in the study and randomly stratified into two treatment groups (test and control) based on CR1 receptor number and body weight. To block iodine uptake by the thyroid, all animals received supersaturated potassium iodide solution orally once daily from day –2 to day 2 (Ross et. al., 1985; Ferguson et.al., 1995).

The animals were tranquilized with tiletamine (A.H. Robbins Co., 6 mg/kg intramuscularly) for initial blood collection, dose administration, and whole body gamma imaging. Tranquilization was performed with ketamine HCl (10 mg/kg intramuscularly) for subsequent blood collection and gamma imaging. Animals were also anesthetized with isoflurane inhalant (1-3%), delivered through a facemask, during the 5-minute gamma imaging procedures. To slow absorption of the study agent and balance the timing of fluid collections, animals received a 25 mL/kg subcutaneous injection of Lactated Ringer’s Solution prior to dosing and following the 8-hour and 24-hour sample collections.
On day 1 of the study, the six cynomolgus monkeys received an intravenous bolus dose of 1.74 mg/kg of infliximab approximately 30 minutes prior to the administration of radiolabeled IgG. At time 0 minutes, test animals 1101, 1102, and 1103 received an intravenous bolus dose of 0.5 mg/kg of 125I-labeled rhesus monkey anti-infliximab IgG, and control animals 2101, 2102, and 2103 received an intravenous bolus dose of 0.5 mg/kg of 125I-labeled rhesus monkey non-immune IgG. A summary of the timing of all sample collections is provided in Table 1. Gamma images of each animal were obtained at 5 minutes and at 1, 8, and 24 hours following treatment with the radiolabeled antibodies. Gamma images at 48 and 72 hours were obtained for the animals remaining in each treatment group.

Test and Control Materials

A 100-mg vial of lyophilized infliximab was reconstituted with 10 mL of USP sterile water for injection. The final protein concentration of 9.68 mg/mL was determined by absorption at 280 nm.

The test antibody (rhesus monkey anti-infliximab) and the control antibody (non-immune rhesus monkey IgG) were both purified by affinity chromatography using a liquid chromatography instrument (AKTA FPLC, Amersham Pharmacia). The test antibody was prepared by immunization of a cynomolgus monkey with infliximab every three weeks until a high titer antiserum specific to the variable region of infliximab was achieved. The control antibody was purified using a Protein G affinity chromatography column; the test antibody was purified using an infliximab affinity column. Affinity columns were equilibrated and loaded.
using modified Dulbecco's phosphate buffered saline (DPBS) and eluted with 100 mM glycine, pH 2.0. The eluted antibodies were dialyzed into borate saline (0.003 M borate, 0.15 M NaCl, pH 7.8), concentrated, 0.2 µM filtered, and stored at 4°C. The antibodies were radiolabeled with 125NaI using the iodogen method (Fraker et. al., 1978). The final 125I-labeled rhesus monkey anti-infliximab IgG had a protein concentration of 1.39 mg/mL and a specific activity of 1.43 x 10⁶ counts per minute (CPM)/µg (652 µCi/mg). The final 125I-labeled non-immune rhesus monkey IgG had a protein concentration of 1.31 mg/mL and a specific activity of 1.35 x 10⁶ CPM/µg (616 µCi/mg based on 100% gamma counter efficiency).

**Plasma Concentrations of Radiolabeled Antibody**

The concentration of the radiolabeled antibodies was determined in plasma samples following trichloroacetic acid (TCA) precipitation. Briefly, 90 µL of serum was precipitated with 10 µL of 100% TCA. Following centrifugation, three 25-µL aliquots of the serum supernatant and the resultant protein pellet were analyzed on a Wallac 1470 Wizard gamma counter. The plasma concentrations of infliximab-specific (test) and non-specific (control) radiolabeled antibodies were reported by converting plasma TCA precipitable CPM to µg/mL equivalents of antibody.

**High-Performance Liquid Chromatography Analysis of Plasma Samples**

Ethylene diamine tetra-acetic acid (EDTA) plasma samples were thawed at room temperature and centrifuged; a 100-µL aliquot of the cleared plasma was dispensed into autosampler vials for analysis on a PerSeptive Biosystems Integral 100Q high-performance liquid chromatography (HPLC) column equipped with a Packard 500TR Series flow scintillation...
analyzer. The sample was separated on a 600 x 7.5-mm BioSep-SEC-S 3000 PEEK (Phenomenex) column using an isocratic 1X DPBS buffer. HPLC analysis was performed for all collected timepoints. The HPLC column was calibrated using molecular weight standards prior to test sample analysis, and least squares regression analysis was performed on the log of the molecular weight of the standard versus its retention time.

Plasma Concentrations of Infliximab

Plasma infliximab concentrations were determined with a sandwich ELISA format using two different, specific murine antibodies to quantify free infliximab concentrations relative to a standard curve (Maini et al., 1998). Theoretically, due to the specificity of the murine antibodies, this assay could possibly detect IFX that was monovalently complexed by anti-IFX, but this is not supported by clinical data. Pharmacokinetic analysis to determine the mean maximum plasma concentration ($C_{max}$), half-life ($t_{1/2}^\alpha$ and $t_{1/2}^\beta$), area under the curve (AUC), clearance (CL) and volume of distribution ($Vz$) for the radiolabeled antibodies as well as for infliximab was completed with non-compartmental techniques using WinNonLin Professional V.3.3 (Pharsight Corp.).

Red Blood Cell Analysis for Radiolabeled Antibodies

To characterize whether the infliximab-test antibody complexes were being eliminated by binding to RBCs, we measured the amount of radioactivity bound to these cells over time for the two groups of animals. The total CPM in the packed RBCs from 200 µL of whole blood was determined for both test and control animal serum. EDTA tubes were immediately placed on ice following blood collection. An aliquot of the whole blood was layered onto an oil mixture
containing eight volumes of dibutyl phthalate (Sigma D2270) and two volumes dinonyl phthalate (Fluka # 80151). The tubes were centrifuged and then frozen on dry ice. The frozen RBC pellet was clipped into polystyrene gamma counter tubes and analyzed on a Wallac 1470 Wizard gamma counter. Based on specific activity, these numbers were converted to number of iodinated molecules bound per RBC.

**CR1 Quantitation by Radioimmunoassay**

The RBCs of the test animals were purified by centrifuging Alsevers (Sigma) diluted whole blood layered onto Ficoll-Paque PLUS (Amersham Pharmacia Biotech). The RBC pellet was washed with 1% BSA/PBS to remove the buffy coat, and an aliquot of 20% erythrocytes (approximately 4 x 10^8 cells) was incubated with ^125^I-7G9 anti-CR-1 mAb. The mixture was layered onto an oil mixture containing eight volumes dibutyl phthalate (Sigma # D2270) and two volumes dinonyl phthalate (Fluka # 80151). The tubes were centrifuged and then frozen on dry ice. The frozen RBC pellet was removed with clippers and placed into a polystyrene tube and analyzed on a Wallac 1470 Wizard gamma counter.

**CR1 Analysis by Flow Cytometry**

Washed RBCs (as defined in previous section) at a concentration of 6 x 10^7 were incubated with Alexa Fluor 488-conjugated 7G9 anti-CR1 antibody or Alexa Fluor 488-conjugated 7B7 isotype control antibody. Following incubation, cells were washed to remove excess 7G9 or 7B7 antibody, resuspended in 1.0% paraformaldehyde, and stored at 4°C until flow cytometry (FacsSCAN) analysis was performed (Ross et al 1985, Ferguson et al 1995).
**Gamma Imaging Studies**

Gamma images for each animal were obtained at 5 minutes and 1, 8, and 24 hours following treatment with radiolabeled antibody. Gamma imaging at 48 and 72 hours was also performed as appropriate for the treatment group. The anesthetized animals were placed in a prone position on the collimator for imaging studies. The field of view contained the region from the neck to lower abdomen of the animal. Following necropsy, gamma images of the intact liver, lungs, kidney, and spleen were also acquired. Activity was determined with a Technicare 410/520 gamma camera, equipped with a large field of view and a medium energy collimator attached to a nuclear medicine computer (Technicare 560). Counts were performed over a 5-minute interval.

**Histology**

Immediately following imaging, sections from the liver, lungs, kidney, and spleen were fixed in 10% neutral buffered formalin. The tissues were trimmed, embedded, and sectioned. Slides were stained with hematoxylin and eosin. Slides were read and interpreted by a veterinary pathologist.

**Immunohistochemistry**

Two, approximately 1.0-cm² sections were collected from the liver, lungs, kidney, and spleen of each animal. The sections were placed in OCT media and stored at −70°C. Ten slides from each piece of tissue (5 µM thick) were prepared. Immunoperoxidase staining (secondary antibody) of tissue sections was performed using anti-infliximab antibody C248 at a dilution of 1:10 for one hour and antibody C465, the parental murine antibody from which infliximab was
derived, at a dilution of 1:100 for 1.5 hours. Endogenous peroxidase was blocked with methanol for 3 minutes, and non-specific background was blocked by incubating sections for 5 minutes in normal swine serum.

_Total Complement Hemolytic Activity_

The complement system represents a group of over 20 serum proteins that may be activated by the presence of immune complexes. Total complement hemolytic activity (CH50) was measured in a standard assay using antibody-sensitized sheep RBCs as substrate (Taylor et al., 1997).
RESULTS

Plasma Concentrations of Radiolabeled Antibody

The mean plasma concentrations of infliximab-specific (test) and non-specific (control) radiolabeled antibodies over time showed low coefficients of variation for both groups at each time point, reflecting the consistency of dosing across groups as well as the in vivo processing. Although comparable amounts of $^{125}\text{I}$ test and control antibodies were administered, notably lower serum concentrations of the immune (test) antibodies were observed relative to the control antibodies at the 5-minute collection point (11.7 µg/mL and 15.3 µg/mL, respectively). The serum concentration of the test antibody decreased more rapidly than that of the control antibody through the first 8 hours and then closely paralleled the level of control antibody through 72 hours. The antibody measurements were based upon the specific radioactivity of the test and control antibodies; therefore, the calculated concentration of these antibodies could reflect a detection of free antibody and/or an antibody-immune complex with infliximab.

High-Performance Liquid Chromatography Analysis of Plasma Samples

HPLC analyses were performed to determine whether immune complexes formed between infliximab and the test or control antibodies. The plasma HPLC radioactivity traces for all three control animals at all time points demonstrated an IgG monomer peak eluting at 16 minutes and a small free $^{125}\text{I}$ peak at 24 minutes retention time. Overlaid traces from a representative control monkey 2101 at 5 minutes and 24 and 48 hours are shown in Figure 1 (top panel). This profile clearly shows that the control $^{125}\text{I}$-monkey IgG did not form a higher molecular weight complex with infliximab.
The plasma HPLC radioactivity retention profiles were similar for the three test animals and distinct from the profiles observed for the control animals. Representative profiles from test monkey 1101 for plasma collected at 5 minutes and 24 and 48 hours are shown in Figure 1 (bottom panel). Peaks were observed at retention times of 12.8, 14.5, 15.6, 17.2, 19.2, and 22 minutes. The peaks at 12.8 and 14.5 minutes are characteristic of the size (retention time) expected for immune complexes of infliximab and $^{125}$I-anti-infliximab, and the peak at 15.6 minutes is consistent with the retention time of uncomplexed $^{125}$I labeled monkey anti-infliximab IgG. The peaks observed at 17.2 and 19.2 minutes are IgG fragments, represent less than 5% of the total protein, and were present in the $^{125}$I labeled monkey anti-infliximab prior to injection. The small peak at 22 minutes is free $^{125}$Iodine. This trace clearly shows that immune complexes were formed \textit{in vivo} by 5 minutes following test antibody administration. These traces also indicate that the largest immune complexes (12.8 minutes) are larger than 670 Kd (based on gel filtration standards), are eliminated the fastest, and are not detectable at 24 hours.

\textit{Plasma Concentrations of Infliximab}

Prior to administration of the radiolabeled antibodies, the mean plasma infliximab concentration was approximately 8 $\mu$g/mL lower for the animals in the test group receiving the immune antibody than for the control animals, and this relative difference was maintained for 8 hours following receipt of the radiolabeled antibodies.

Since the duration of PK sample collection was variable (24, 48, or 72 hours) for each of the three animals in each treatment group, the derived pharmacokinetic data analysis for the radiolabeled antibodies as well as for infliximab was performed using the average plasma
antibody concentrations at each time point. The mean Cmax, t1/2α, t1/2β, AUC, CL, and Vz values are provided in Table 2.

Red Blood Cell Analysis for Radiolabeled Antibodies

Elimination of infliximab antibody complexes via RBCs, as measured by RBC-bound radioactivity for test and control groups, is shown in Figure 2 (top panel). Comparable amounts of test and control antibodies were bound to the RBCs immediately following administration of the radiolabeled antibodies. Lower levels of test antibody binding were observed from 24 to 72 hours relative to the control antibody, reflecting the lower plasma concentration for the test antibody.

The percentage of the total radioactivity from 1.0 mL of whole blood that was bound to the RBCs was compared between the test and control groups in Figure 2 (bottom panel). Even though the test group had fewer molecules of iodinated antibody binding per RBC (as shown in Figure 2, bottom panel), the percentage of the total activity binding to the RBCs was higher in the test group than in the control group.

CR1 Quantitation by Radioimmunoassay and Flow Cytometry

To evaluate whether CR1 receptors were being stripped from the RBCs as a result of immune complex binding and clearance, RBC CR1 receptor analyses were performed using both radioimmunoassay (RIA) and flow cytometry. The RIA analysis provides a quantitative assessment of the mean CR1 receptor number for individual RBC samples. The total number of CR1 receptors per RBC remained consistent over time for all animals tested with no significant change in CR1 receptor number between the test and control groups for all time points combined (p=0.452).
The availability and homogeneity of the CR1 receptor number was also qualitatively assessed within the RBC population using flow cytometry. All baseline samples (obtained at -40 minutes) for all animals had similar signal intensity when stained with the anti-CR1 receptor antibody 7G9, as shown by the open peak (Figure 3). The shaded peaks show the nonspecific signal observed with the isotype control antibody. Overall the flow cytometry data did not indicate consistent treatment group differences. Both groups of animals showed a small decrease in anti-CR1 receptor binding at 0.25 and 0.5 hours, with some shifts in the population profile over time. It was expected that if immune complexes were binding to CR1 receptors, the test group of animals would show a more notable reduction in binding of the 7G9 antibody relative to the control group. Taken together, the RIA and flow cytometry results did not indicate that CR1 had a role in binding and elimination of infliximab and anti-infliximab immune complexes. On a strict stoichiometric basis, the injection of approximately \(7 \times 10^{15}\) antibody molecules (based on a total injection of 1.75 mg of test antibody) should have been enough to saturate the monkey’s \(1 \times 10^{15}\) CRI receptors (based on a 3.5 kg monkey, a blood volume of 65 mL/kg, and \(5 \times 10^9\) RBCs/mL of blood). However, based on HPLC traces, the immune complexes formed were of variable sizes and therefore the total number of immune complexes was probably less than \(7 \times 10^{15}\). Therefore, it is not known whether injection of higher concentrations of test antibody would have resulted in more C3b opsonized immune complex capable of resulting in a reduction in CR1.
Gamma Imaging Studies

Gamma images of each animal were obtained at 5 minutes; 1, 8, and 24 hours; and at the time of necropsy. Gamma images for a representative test and control animal at 24 hours are shown in Figure 4. The test animals had a higher concentration of radioactivity in the liver, while the radioactivity in the control animals appeared to be distributed throughout the blood pool, with the highest concentration of radioactivity observed in the heart area. Following necropsy at 24, 48, and 72 hours, gamma images of the excised intact liver, lungs, kidney, and spleen were obtained for each animal. The radioactivity of each organ, as a proportion of the whole body CPM at the time of necropsy, was determined. The mean percentage of total radioactivity in the lung, liver, spleen, and kidney for the test group was 2.2%, 8.1%, 0.6%, and 2.0%, respectively. Respective percentages for the control group were 3.3%, 6.6%, 0.4%, and 1.0%. There was a significantly higher percentage of counts for the test group compared with the control group for the liver (p=0.032) and the kidneys (p=0.010).

Histology

Histology slides of the lung, liver, kidney, and spleen were prepared from each animal at the time of necropsy and interpreted by a licensed veterinary pathologist. There were no reported macroscopic or microscopic findings from any of the examined organs in either treatment group.

Immunohistochemically, infliximab was detected using a specific monoclonal antibody in the cytoplasm in the liver Kupffer cells, kidney endothelial cells, and the reticular mononuclear cells in the germinal center of B-cell follicles in the white pulp of the spleen in all test animals at all time points. There was some staining in the spleen of the control animal necropsied at 24 hours, but to a lesser extent than the test animals. Anti-infliximab IgG was detected in
hepatocytes, kidney endothelial cells in the glomerulus, and in mononuclear phagocytes in the sinusoids in the red pulp of the spleen in all test animals at all time points. Representative immunohistochemistry sections are shown in Figure 5.

**Complement System Concentration: CH50 Levels**

Figure 6 shows the mean CH50 levels (U/mL) for the test and control groups at –40 and 30 minutes and 3, 24, 48, and 72 hours. There was no difference in the mean CH50 level between test and control groups prior to initiation of treatment (p=0.832). Following administration of radiolabeled antibody, the mean CH50 level in the test group remained lower than in the control group until the levels equilibrated at 72 hours. In the test group, there was a marked decrease in the CH50 level at 30 minutes; however, this was not significantly different than in the control group (p=0.232). The overall gradual increase in CH50 values for both the test and control groups is likely a result of stress on the animals due to anesthesia, handling, and repeated blood sampling.

**Hematology and Serum Chemistry**

The results of hematology and serum chemistry analyses were comparable for the test and control treatment groups and within normal reference ranges (data not shown). However, Figure 7 shows there was a significant mean increase in serum glucose levels, compared with pretreatment values, for the test group compared with the control group (52.7 and 13.7 mg/dL, respectively p=0.025).
DISCUSSION

Currently, there are only limited data available regarding how immune responses directed
against therapeutic proteins influence clearance of therapeutic proteins. This study provides
novel information regarding the formation and size of immune complexes formed with a
therapeutic monoclonal antibody, whole body distribution of the complexes, and the rate and
mechanism of their elimination from circulation. This study also provides information on how an
immune response can affect the clearance and half-life of a therapeutic monoclonal antibody and
a perspective on the relevance of assays to detect an immune response in the presence of
detectable therapeutic protein.

This study was designed to model the elimination of an immune response in the presence
of excess antigenic protein. The administration of a therapeutic amount of infliximab
(approximately 2 mg/kg, resulting in 30-50 µg/mL of infliximab), followed by injection of a
limited, but physiologically relevant amount of test ¹²⁵I-labeled anti-infliximab antibody
(resulting in approximately 10 µg/mL of test antibody at 5 minutes post-injection) in cynomolgus
monkeys resulted in rapid in vivo formation of immune complexes of variable size. The immune
complexes had a $t_{1/2\alpha}$ of approximately 10 hours and a $t_{1/2\beta}$ of approximately 37.5 hours, with
the highest molecular weight complexes being preferentially eliminated. Immune complexes did
not form between infliximab and radiolabeled non-immune control antibody.

The mean terminal half-life of infliximab in this study was estimated to be 4.4 days in
test animals and 5.0 days in control animals. This indicates that, as expected, immune complex
formation resulted in accelerated elimination of a fraction of the circulating infliximab, which
was reflected in the lower serum concentrations observed at all time points in the test group. The \( \beta \)-half-life of the remaining infliximab in the circulation of the test animals was not significantly different from the values observed in control animals. These results are consistent with removal of only a portion of the circulating infliximab via the administered immune antibody. It is expected that the \textit{in vivo} production of an immune response would result in a greater and more sustained reduction in circulating infliximab concentrations. The limited effect of low-level antibody responses on circulating infliximab levels is supported by recently published clinical data based on a cohort of 125 infliximab-treated Crohn’s disease patients (Baert et al., 2003). In that study, patients with low concentrations of the anti-infliximab antibody (i.e., <8.0 \( \mu g/mL \)) were found to have a median duration of response of 71 days, and this low level response did not affect the circulating infliximab concentration.

In designing this study, complement-mediated and Fc receptor-mediated immune complex clearance mechanisms were considered. In a complement-mediated mechanism, immune complexes bind to the CR1 receptor on RBCs via complement component C3b. The erythrocyte traverses the liver where the immune complex bound to CR1 is recognized by Fc receptors (most likely Fc gamma receptor RI) on acceptor cells and phagocytized. The erythrocyte, now stripped of immune complex and CR1 receptors, then returns to the circulation (Cornacoff et al., 1983; Taylor et al., 1997). If the immune complexes are not sufficiently opsonized with complement they can still be cleared in an Fc receptor mediated mechanism. In the complement-independent Fc receptor-mediated mechanism, immune complexes bind to macrophages via the immunoglobulin Fc region and the complexes are then phagocytized or
pinocytized depending on whether they are particulate or soluble (Daeron, 1997; Aderem et al., 1999).

In this study, the RBCs appeared to play only a limited role in elimination of these immune complexes, and it did not appear that CR1 participated in the mechanism by which the complexes were eliminated. The peak binding activity of 5.2% in the test group occurred at 8 hours. This percentage is comparable to results of a recent study in which immune complexes were identified that were very poor at complement fixation (Davies et al., 2002). In fact, complexation of the antibodies to the therapeutic antigen (antibody) can promote binding to the low affinity FcγRII or FcγRIII receptors (Daeron, 1997). Kupffer cells and liver endothelial cells both possess FcγRII and FcγRIII receptors (Muro et al., 1993; Ahmed et al., 1995; Lovdal et al., 2000). Once the immune complex has been engaged by the Fcγ receptors, the complexes would be internalized and travel from the endosome to the lysosome (Lovdal et al., 2000), where the complexes would be expected to be enzymatically digested.

Gamma imaging showed a higher proportion of the total radioactivity detected in the excised liver and kidney from the animals in the test group compared with those in the control group. In addition, the gamma imaging results were confirmed immunohistochemically since both infliximab and anti-infliximab IgG were independently detected in the liver and kidneys as well as in the spleen of the test animals. These results are consistent with immune complexes of infliximab and anti-infliximab being efficiently taken up by the mononuclear phagocytic system in the liver, followed by degradation and excretion of radio-iodinated species in the urine.
(Johansson et al., 2002). The spleen involvement is not as clear but perhaps suggests immune surveillance.

The increase in serum glucose levels, compared with pretreatment values, for the test group compared with the control group may be explained by the release of prostaglandins from Kupffer cells, which have been shown to increase the level of hepatic glycogenolysis (conversion of glycogen into glucose) following immune complex stimulation (Hespeling et al., 1995). However, these trends were not observed in a recent human clinical study of 1049 patients with early RA in which 67 subjects tested positive for antibodies to infliximab. Urine glucose test results were available for 63 of these subjects at 30 weeks following the initiation of infliximab treatment and all results were within normal reference ranges (data on file).

Immune complexes formed between a biotherapeutic agent and either neutralizing or non-neutralizing antibodies may be undetectable because of rapid in vivo clearance. In this study, we have shown that immune complexes with a therapeutic monoclonal antibody were rapidly and safely eliminated in a monkey model.

These data indicate that it may be difficult to measure antibodies in the presence of excess biotherapeutic protein because the immune complexes are eliminated fairly rapidly. A variety of factors will affect the detection of the immune antibodies in the presence of excess therapeutic monoclonal antibody including the concentration, affinity, and rate of production of the immune antibody, the dosing and pharmacokinetic profile of the therapeutic monoclonal antibody, the assay sensitivity for the immune antibody, and the sampling schedule. It appears
that if immune complexes are present, they would probably represent only a fraction of the amount of immune antibody produced. Therefore, although this cannot always be achieved for subjects receiving long-term or chronic treatment, the best assessment of immune antibodies would be conducted after longer follow-up and in the absence of detectable therapeutic monoclonal antibody.
ACKNOWLEDGMENTS

We are thankful to Peter Bugelski for useful discussions and to Mary Whitman and Scott Newcomer for their valued assistance in the preparation and submission of this manuscript.
REFERENCES


FOOTNOTES

Centocor Inc. has provided financial support for this manuscript.

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FIGURE LEGENDS

**Figure 1:** HPLC analysis of plasma from control monkey 2101 (top panel) and test monkey 1101 (bottom panel) at 5 minutes, 24 hours, and 48 hours

**Figure 2:** The top panels show the mean number of molecules of $^{125}$I-labeled antibody bound per RBC, and the bottom panel shows the percent of the total radioactivity in whole blood associated with RBCs for both the test and control groups. These data indicate that there is some preferential binding of the test antibody to the RBC, even though the absolute number of molecules bound per RBC is higher for the control group.

**Figure 3:** Flow cytometry analysis of red blood cell Complement Receptor 1 (CR1). Histogram of test animal 1101 at – 40 minutes (right) and 24 hours (left). Anti-CR1 antibody 7G9 binding is shown in green and the isotype control antibody 7B7 binding is shown in purple.

**Figure 4:** Gamma images for test animal 1101 and control animal 2101 at 24 hours.

**Figure 5:** Immunohistochemical staining of liver (400X, upper right) and spleen (200 X, upper left) with antibody C248 and the kidney (400X, lower right) and spleen (200 X, lower left) with antibody C465. The upper left panel shows a B-cell follicle of the white pulp of the spleen with
positively staining cells in the center. The lower right panel shows positively staining endothelial cells in a glomerulus while the tubules are negative.

**Figure 6:** Mean CH50 values for test and control animals

**Figure 7:** Serum glucose levels 48 hours after administration of radiolabeled antibodies in test monkeys 1101, 1102, and 1103 and control monkeys 2101, 2102, and 2103
<table>
<thead>
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<th>Serum Chemistry</th>
<th>CH50$^*$</th>
<th>Blood for Sponsor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to initiation of study</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1 at –40, 5, 15, and 30 minutes; 1, 3, 8, 24, and 48 hours following the $^{125}$I-labeled dose</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Day 1 at –15 minutes</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Day 1 at –40, -15, and 30 minutes; 3 and 24 hours following the $^{125}$I-labeled dose</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Prior to necropsy</td>
<td>X$^{**}$</td>
<td>X</td>
<td>X</td>
<td>X$^{**}$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume of Whole Blood/ Time point</th>
<th>0.75 mL</th>
<th>1.8 mL</th>
<th>0.25 mL</th>
<th>1.0 mL</th>
<th>3.0 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticoagulant</td>
<td>EDTA</td>
<td>None</td>
<td>None</td>
<td>EDTA</td>
<td>EDTA</td>
</tr>
</tbody>
</table>

$^*$ Serum shipped to AniLytics (Gaithersburg, MD) for analysis.

$^{**}$ Sample collected only if the necropsy was performed after the 48-hour time point based on gamma imaging.
**Table 2:** Pharmacokinetic parameters of $^{125}$I-labeled antibodies and infliximab in test and control animals

<table>
<thead>
<tr>
<th></th>
<th>$t_{\frac{1}{2}a}$ (hours)$^a$</th>
<th>$t_{\frac{1}{2}b}$ (hours)$^a$</th>
<th>Cmax ($\mu$g/ml$^a$)</th>
<th>AUC 0-24 hours (hr. $\mu$g/mL)</th>
<th>CL$^b$ (mL/kg/day)</th>
<th>Vz (mL/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-Ab (test)</td>
<td>10.1</td>
<td>37.5</td>
<td>11.6</td>
<td>96.3</td>
<td>77.0</td>
<td>71.8</td>
</tr>
<tr>
<td>$^{125}$I-Ab (control)</td>
<td>22.1</td>
<td>86.5</td>
<td>15.3</td>
<td>235</td>
<td>13.5</td>
<td>42.7</td>
</tr>
<tr>
<td>Infliximab (test)</td>
<td>27.7</td>
<td>105</td>
<td>37.7</td>
<td>546</td>
<td>14.0</td>
<td>59.2</td>
</tr>
<tr>
<td>Infliximab (control)</td>
<td>21.8</td>
<td>119</td>
<td>46.0</td>
<td>656</td>
<td>11.5</td>
<td>46.3</td>
</tr>
</tbody>
</table>

- a. Half-life based on mean concentration values.
- b. Clearance determined over 72 hours.
- c. Data represent the infliximab and anti-infliximab immune complex.
Figure 1

Control Monkey 2101

Test Monkey 1101

Retention Time (minutes)

CPM

Retention Time (minutes)

CPM

--- 5 min  24 hrs  --- 48 hrs

--- 5 min  24 hrs  --- 48 hrs
Figure 2

The figure shows the mean number of molecules per RBC over time (Hours) and the percent of total CPM over time (Hours) for Test and Control groups. The data points are represented with markers for Test (diamonds) and Control (squares). Error bars indicate the variability of the data. The y-axis for mean number of molecules per RBC ranges from 0 to 1000, while the y-axis for percent of total CPM ranges from 1% to 6%.
Figure 3
Figure 4

[Image of a figure with labeled parts: HEAD, LIVER, and HEART]
Figure 6

![Graph showing mean CH50 (units/mL) over time for Test and Control groups.](image)
Figure 7

![Glucose levels in Cynomolgus Monkeys](image)

- **Baseline**
- **Necropsy**

**Glucose**

- mg/dL

**Cynomolgus Monkey**

- T-1101
- T-1102
- T-1103
- C-2101
- C-2102
- C-2103