Figure S1: Incomplete inhibition of ADA to infliximab is not caused by ADA-drug complexes. A) FPLC fractionation of patient serum. To eliminate drug-ADA complexes, fractions corresponding to monomeric IgG were pooled and used to repeat the TNF competition assay. B) Percentage of AU that is inhibited in binding radiolabeled infliximab Fab' after adding 5000 ng of TNF. No significant difference is detected between patient serum and the monomeric IgG fraction. Open dots: patient showing complete inhibition, closed dots: patients showing incomplete inhibition in TNF competition assay, ns = not significant using paired t-test.
Figure S2: There is no relationship between the percentage of neutralizing antibodies and the level of ADA. The percentage of units that is inhibited in binding radiolabeled drug Fab’ after adding 5000 ng of TNF, and the ADA level determined by our diagnostics department. Each symbol represents one patient.
MATERIALS AND METHODS

Patient material
Patient sera from our diagnostic department were selected for ADA positivity for either adalimumab, golimumab, certolizumab, or infliximab measured using the standard antigen binding tests (ABT). In total, serum of 68 patients (adalimumab (n=21), golimumab (n=4), certolizumab (n=9) and infliximab (n=34)) send in to our diagnostic department were analyzed. In all cases, the amounts of antibodies exceeded 100 AU/ml. Of these patients, no further clinical data is available. In case of infliximab, adalimumab and golimumab, no free drug was detected in the serum samples. On the other hand, most certolizumab samples contained free drug. However, since certolizumab is a Fab fragment and does not bind to protein A, free drug will be washed out during the ABT and therefore will not interfere with antibody measurements[1].

Antigen binding test (ABT) and TNF competition assay
To determine the neutralizing capacity of ADA, the TNF competition assay was used. First, the optimal serum dilution was determined for every patient using the ABT, an assay routinely used by our diagnostics department, validated for each TNF-blocker. Patient serum was serially diluted in PBS/0.3% bovine serum albumine (BSA, Millipore). Fifty μl of diluted serum (or 50 ul of the optimal concentration in case of the TNF competition assay) was added to 1 mg of Sepharose-immobilized protein A (GE Healthcare) in PBS-AT (PBS containing 0.3% BSA, 0.2% Tween-20 (Merck) and 0.01M EDTA) in a final volume of 800 μl and incubated overnight on a rotator. The samples were subsequently washed five times with washing buffer (PBS/0.005% Tween-20) and incubated with 125I labeled Fab’ fragments of adalimumab, certolizumab or infliximab, or biotinylated golimumab Fab’ (no significant difference was observed between directly radiolabeled drug or indirect radiolabeling via biotinylation), all diluted in PBS-FAT (PBS-AT containing 40 μg/ml IVIG F(ab’)2, Sanquin) in order to avoid false-positive results due to anti-hinge antibodies[2]. In case of the TNF competition assay, the labeled drugs were preincubated with a titration of TNF (Human TNF-alpha cct premium, Active Bioscience), ranging from 0-5000 ng per tube. Labels were added in a final volume of 800 ul, and incubated for 5 h. Unbound labeled Fab’ was washed away and in case of golimumab 125I labeled streptavidine was added, incubated overnight and subsequently washed. Sepharose-bound radioactive label was measured using Wallac 1260 Multigamma II (LKB). Sample measurements were normalized to the percentage of total radioactively labeled input (% binding). For the TNF competition assay, the % binding was converted into arbitrary units (AU) using a standard curve (pooled serum of ADA-positive patients
for adalimumab and infliximab, polyclonal rabbit-anti-drug for certolizumab and
golimumab). Percentage of inhibition was calculated by dividing the AU of inhibited
samples by the AU of non-inhibited samples.

**WEHI bioassay**
The biological activity of ADA was analyzed using the TNF-sensitive WEHI bioassay.
Per well 40,000 WEHI-164 cells were plated in IMDM (BioWhittaker) containing 5% FCS
(Bodinco), 100 U/ml penicillin, 100 μg/ml streptomycin (both from Gibco), 1 μg/ml
Actinomycin D and 50 μM β-Mercapto-ethanol (both from Sigma). Subsequently, a
mixture of 100 pg/ml TNF (Strathmann), 30 ng/ml infliximab or adalimumab and a
titration (twofold dilution, 1:100 – 1:6400) of sera containing ADA against infliximab or
adalimumab were added. As a positive control, a titration of rabbit-anti-infliximab or
rabbit-anti-adalimumab was added to the WEHI cultures containing TNF and infliximab
or adalimumab, respectively. WEHI-164 cells were incubated for 24 h at 37°C and 5% CO₂
after which cell viability was measured using the MTT-reduction method. MTT
(Sigma, diluted in 0.14M NaCl and 0.01M HEPES) was added to the cell cultures in a
final concentration of 0.83 mg/ml and incubated for four hours, after which SDS (Gibco,
diluted in 0.01M HCl) was added to a final concentration of 4% for overnight incubation.
Adsorption was measured at 595 nm and as reference 670 nm using the Multiskan EX
(Thermo Scientific).

**Monomer isolation by FPLC**
Serum of five patients was fractionated by Fast protein liquid chromatography (FPLC) to
isolate fractions that contain monomeric IgG but no complexes of two or more IgG
molecules. Serum was three times diluted in PBS and filtered (0.22 um filter, Merck
Millipore) prior to application. A Superdex 200 10/300 GL column in combination with the
FPLC system GE ÄKTA Explorer (Amersham Pharmacia) was used to fractionate the
sera. Molecular weight estimations were made using intravenous immunoglobulin (IVIG,
20 mg/ml) that contains both monomeric and dimeric IgG. Fractions of patient serum
containing only monomeric IgG were pooled for each patient.

**TNF/mouse IgG competition assay**
Further characterization of the target of non-neutralizing ADA to infliximab was done
using the TNF competition assay with addition of polyclonal BALB/c mouse IgG
(Molecular Innovations). Per patient, twelve tubes containing 1 mg of CaptureSelect anti-
human IgG-Fc Affinity Matrix (Life Technologies) were incubated overnight with 50 μl of
the optimal serum dilution. After washing, 20 ug mouse IgG in PBS-FAT was added in
six of the tubes, whereas in the other six tubes only PBS-FAT was added. In both sets of
tubes, 50 ul of $^{125}$I labeled infliximab Fab' preincubated with a titration of TNF, ranging
from 0-5000 ng per tube, was added. The tubes were incubated for 5h on a rotator and
subsequently washed. Bound radioactivity was measured as described above.

REFERENCES


2. Rispens T, de Vrieze H, de Groot E, et al. Antibodies to constant domains of
therapeutic monoclonal antibodies: anti-hinge antibodies in immunogenicity