ABSTRACT: A substantial number of patients enrolled in clinical studies of TNFR55-IgG1 in TNF-neutralizing treatment of rheumatoid arthritis and multiple sclerosis developed antibodies to the recombinant human protein. To enable more detailed investigation subgroups of patients donated small blood samples. TNFR55-IgG1 reactive antibodies were affinity purified from plasma; IgM and IgG class antibodies reactive with TNFR55-IgG1 were found which varied considerably in titer and kinetics of appearance among individual patients. The affinity purified antibody fractions included specificities to the receptor moiety of TNFR55-IgG1, but also rheumatoid factor and other pre-existing antibodies directed to the IgG1 moiety. The antibodies bound to Fc receptors, but not detectably to TNFR55 at the human cell surface. No agonistic or neutralizing activities of these antibodies were detected. Major linear epitopes clustered in the TNFR55 sequence in close proximity to the IgG1 fusion site. The relative content of antibodies to linear and conformational epitopes was highly variable among patients. Route and frequency of administration rather than underlying disease appeared to influence the major linear B cell epitopes selected. Human Immunology 60, 774–790 (1999). © American Society for Histocompatibility and Immunogenetics, 1999. Published by Elsevier Science Inc.

KEYWORDS: TNF-R-IgG fusion protein; clinical trial; rheumatoid factor; autoimmunity; epitope mapping

ABBREVIATIONS

MS Multiple Sclerosis
RA Rheumatoid Arthritis
TNFR-IgG1 TNF receptor-IgG1 fusion protein (Lenercept®)
iv intravenous
sc subcutaneous
HRP horseradish peroxidase
AP alkaline phosphatase
PE phycoerythrin
TMB 3,3',5,5'-tetramethylbenzidine
RF rheumatoid factor
MTP microtiter plate
RT ambient temperature
ECF enhanced chemiluminescence
FcyRI Fcy receptor I
IC50 50% inhibitor concentration
HUVEC human umbilical vein endothelial cells
INTRODUCTION

Various anti-TNF antibodies and recombinant soluble TNF receptor protein constructs which bind and neutralize tumor necrosis factor (TNF) have been developed for the treatment of rheumatoid arthritis (RA) and multiple sclerosis (MS) patients (for reviews see [1–3]). In clinical studies of RA- and MS-patients, we investigated the antibody response to a recombinant TNF receptor-immunoglobulin fusion protein linking the 55kDa TNF receptor extracellular domain and IgG1 hinge and C2/C3 constant domains (TNFR55-IgG1) [4–11]. TNFR55-IgG1 contained the entirely human sequences of the fusion partners placed next to each other without inserted linker, and was produced in eucaryotic cells. When studied according to protocol, specific anti-TNFR55-IgG1 immuno-reactivities in the plasma of a substantial number of patients after the first few doses were found in a routine double-antigen assay; these and clinical outcome data have been reported elsewhere [6–12]. Subgroups of patients consented to donate small amounts of blood to allow a more detailed investigation of this potential auto-antibody response. Antibodies reactive with TNFR55-IgG1 of both IgM and IgG class were found. Agonistic and neutralizing activities of the antibodies were not detected. Mapping of linear epitopes identified immunodominant sequence clusters which are located within the TNF-receptor moiety in close proximity to the fusion site. Antibody fractions reactive with TNFR55-IgG1 included also rheumatoid factor (RF) and other preexisting antibodies directed to the IgG1 moiety. The major linear B cell epitopes of TNFR55-IgG1 appeared to be determined by route and/or frequency of administration irrespective of underlying disease, RA or MS.

MATERIALS AND METHODS

Patients

Patients participated in the investigations according to protocol-defined procedures. All studies were approved by the institutional review boards of Roche and the participating centers, and all subjects gave informed consent. RA*-patients received a total of 200 mg TNFR55-IgG1 iv either in 3 doses (100 mg loading, followed by two 50 mg maintenance doses every four weeks) or in 6 doses (100 mg loading, followed by five 20 mg maintenance doses every two weeks). RA*-patients received 13 doses of either 50, 25, 12.5, 5 mg TNFR55-IgG1 or placebo sc in weekly intervals. MS*-patients received 7 doses of either 100, 50, 10 mg TNFR55-IgG1 or placebo iv every four weeks. In all three studies blood was drawn 4 weeks after the last dose from patients of selected centers. In a small number of RA*-patients a further specimen was obtained 6 months after the last dose. Blood was collected under sterile conditions and anticoagulated with 10 U/ml Liquemin. Blood was either sent from study centers to Basel and plasma obtained (400 × g for 15 min at 20°C) within 24 h or plasma was obtained on site (400 × g for 15 min at 20°C) and was sent to Basel on dry ice. For some selected RA*-patients, small parts of plasma specimens collected according to study protocol throughout the treatment period were obtained.

Materials

Cell lines. HUVEC were prepared as previously reported [13]; HL60 cells (ATCC CCL 240) were from American Type Culture Collection, Rockville, MD; KYM1-ID4 (KD4) cells were from NIBSC, South Mimms, UK (courtesy Dr. A. Meager).

Antibodies. Anti-TNFR55 (CD120a) mAb, htr-9, anti-TNFR75 (CD120b) mAb, utr-1, and affinity purified rabbit anti-TNFR55-IgG1 antibodies were previously reported [14]. Mouse anti-human ICAM-1 mAb were from British Bio-technology Products Ltd., Abingdon, UK; horseradish peroxidase (HRP)- and alkaline phosphatase (AP)-conjugated anti-human and anti-mouse IgG were from Bio-Rad Laboratories, Hercules, CA; phycoerythrin (PE)-conjugated streptavidine and PE-conjugated goat anti-mouse IgG were from Southern Biotechnology Associates Inc., Birmingham, AL; AP-conjugated streptavidine was from Pharmingen, San Diego, CA; HRP-conjugated streptavidine was from Immunotech, Marseille, France.

Reagents. TNFR55-IgG1 (Lenercept2, Ro 45-2081) was previously reported [4, 5]; Liquemin was from Roche Pharma AG, Reinach, Switzerland; CNBr-activated Sepharose-4B was from Pharmacia Biotech, Uppsala, Sweden; Heparin, Thimerosal, DMSO, α-Phenylenediamine, human IgG and IgM, biotin-amidocarpoate-N-hydroxysuccinimide ester (Biotin-NHS), and BSA were from Sigma Chemical Co., St. Louis, MO; Tween-20 was from Bio-Rad Laboratories, Hercules, CA; M199 and MEM10 medium, heat inactivated FCS, and stock solutions of EDTA-trypsin, Penicillin/Streptomycin and L-Glutamine were from GIBCO BRL/Life Technologies, Paisley, UK; endothelial cell growth supplement (ECGS) was from Collaborative Biomedical Products, Bedford, MA; Crystal violet and 3,3',5,5'-tetramethylbenzidine (TMB) were from Fluka, Buchs, Switzerland; SYPRO-orange was from Molecular Probes, Eugene, OR; AttoPhos (enhanced chemifluorescence (ECF)-reagent) was from Amersham, Little Chalfont, UK.
Analytical Assays

RF was determined by Institute Dr. Viollier, Basel, Switzerland using the N-Latex-RF diagnostic assay (Behringwerke AG, Marburg, Germany). TNFR55-IgG1 reactive antibodies were first screened by a “double antigen” assay [15]. Briefly, 96 well NUNC Immuno-plate “Maxisorp” microtiter plates (MTP) were coated with TNFR55-IgG1 (1.0 μg/ml in 100 mM NaHCO3, pH 9.5), incubated overnight at ambient temperature (RT), washed (H2O, 3×), and blocked with BSA (10 mg/ml BSA in 200 mM Tris/HCl pH 7.5, 0.02% (M/W) Kathon) for at least 24 h at RT. Blocking buffer was removed and 200 μl patient serum (1:5, 1:25, and 1:125 in 100 mM Tris/acetate pH 7.0, 10 mg/ml BSA, 1 mg/ml phenol, 0.02% (M/W) Kathon) or an affinity purified rabbit anti-TNFR55-IgG1 control antibody [15] were added, followed by 50 μl peroxidase-coupled TNFR55-IgG1 (400 ng/ml in 100 mM Tris/acetate pH 7.0, 10 mg/ml BSA, 1 mg/ml phenol, 0.02% (M/W) Kathon). MTP were sealed and incubated for 16–24 h at 7.0, 10 mg/ml BSA, 1 mg/ml phenol, 0.02% (M/W) Kathon) for at least 24 h at RT. Blocking buffer was removed and 200 μl patient serum (1:5, 1:25, and 1:125 in 100 mM Tris/acetate pH 7.0, 10 mg/ml BSA, 1 mg/ml phenol, 0.02% (M/W) Kathon) or an affinity purified rabbit anti-TNFR55-IgG1 control antibody [15] were added, followed by 50 μl peroxidase-coupled TNFR55-IgG1 (400 ng/ml in 100 mM Tris/acetate pH 7.0, 10 mg/ml BSA, 1 mg/ml phenol, 0.02% (M/W) Kathon). MTP were sealed and incubated for 16–24 h at 2–8°C. After washing (6–8×) with PBS containing 0.1% Tween 20, 200 μl TMB/H2O2 substrate (30 mM potassium citrate pH 4.1, 0.5 mM TMB, 2.0 mM H2O2) were added and MTP were incubated 5–15 min at 15–25°C. The peroxidase reaction was stopped with 100 μl 1 M H2SO4 and OD450nm was measured within 1 h with a spectrophotometer. The presumed anti-TNFR55-IgG1 antibody signals were normalized in units of mass equivalence (EU/ml) to a affinity purified rabbit anti-TNFR55-IgG1 antibody standard.

Purification of Anti-TNFR55-IgG1 Antibodies from Human Plasma

Analytical precipitation of anti-tnfr55-igg1 antibodies. 25 μl plasma in 1 ml binding-buffer (PBS containing 0.5 M NaCl, 0.1% Tween-20, 0.1% NaN3), were precleared with Sepharose-4B beads (1 h at RT) and centrifuged (100 × g, 2 min). Supernatant was incubated with 20 μl TNFR55-IgG1 coupled Sepharose-4B beads and overnight at 4°C with gentle agitation. Parallel control precipitations were carried out with uncoupled Sepharose-4B beads. Beads were washed (2×) with 1 ml binding-buffer, bound antibodies were eluted with 20 μl elution-buffer (100 mM glycine pH 2.6, 100 mM NaCl) and 2 μl 1 M Tris/HCl pH 8.0 were added. Eluted proteins were analyzed by 15% SDS-PAGE. The gel was stained with SYPRO-orange, fluorescence was read on a STORM 840 fluorimeter (Molecular Dynamics) (extinction 450 nm; emission was detected using a 520 nm long pass filter) and the obtained signals of proteins bands were quantified using ImageQuant software (Molecular Dynamics). Ig μ- and γ-chain background obtained by precipitation with uncoupled beads was subtracted and band intensities were normalized to IgM and IgG standards on the same SDS-PAGE.

Purification of anti-TNFR55-IgG1 antibodies. 7–8 ml plasma were diluted with 30 ml binding-buffer, preadsorbed to 1 ml Sepharose-4B (1 h at RT), and applied to a 1 ml TNFR55-IgG1-coupled Sepharose-4B affinity column. The column was washed with binding buffer until baseline OD280nm (spectrophotometer, Pharmacia) was reached. Anti-TNFR55-IgG1 antibodies were eluted with elution buffer. Fractions of 900 μl were collected in tubes containing 100 μl 1 M Tris- HCl, pH 8.0, and analyzed by 15% SDS-PAGE. Fractions containing immunoglobulin were pooled and dialyzed against PBS overnight at 4°C.

Biotinylation of Antibodies

1 ml patient anti-TNFR55-IgG1 antibodies or htr-9 (1 mg/ml) were dialyzed in a Slide-A-Lyzer cartridge (Pierce) twice against 1 M NaHCO3 (100 mM, pH 8.2) for at least 4 h at 4°C. 20 μl Biotin-NHS (10 mM in DMSO) were directly injected into the cartridge. After incubation (1 h, at RT) excess Biotin-NHS was removed by dialysis against 1 PBS for 4 h at 4°C.

Binding of Anti-TNFR55-IgG1 Antibodies to Immobilized TNFR55-IgG1 (ELISA)

MTP were coated with 10 ng TNFR55-IgG1/well in 100 μl 100 mM carbonate buffer, pH 9.6, overnight at 4°C. MTP were washed (4×) with 200 μl PBS, and unspecific binding sites were blocked with blocking buffer (2% dry milk powder in PBS, 0.02% Thimerosal) for 90 min at 37°C. MTP were rinsed with washing buffer (PBS, containing 0.05% Tween 20), and 200 μl/well of either biotinylated patient anti-TNFR55-IgG1 antibodies (5 μg/ml) or biotinylated htr-9 (0.5 μg/ml) in blocking buffer containing various concentrations of either TNFR55-IgG1 or human IgG were added. MTP were incubated for 90 min at 37°C. After washing (3×) with washing buffer and addition of 200 μl HRP-conjugated steptavidin (1:5000 in blocking buffer), MTP were incubated for 90 min at 37°C. After washing (3×) with washing buffer and once with PBS, 100 μl peroxidase substrate (0.4 mg/ml 1,2-phenylenediamine in 0.1 M citric acid, pH 5.0, 0.1% H2O2) was added. The reaction was stopped with 100 μl 2 M H2SO4 after a clear color development had appeared (10–15 min) and OD488nm was measured with a Dynatech MR5000 spectrophotometer (Dynatech Laboratories, Billinghurst, England).
Binding of Anti-TNFR55-IgG1 Antibodies to HL60 Cells

50 μl containing 10^5 HL60 cells were incubated with 50 μl of either purified biotinylated patient anti-TNFR55-IgG1 antibodies (10 μg/ml) or biotinylated htr-9 (10 μg/ml) in presence or absence of either TNFR55-IgG1 (500 μg/ml), human IgG (500 μg/ml) or mouse anti-human CD64 mAb (3 μg/ml) in FACS-buffer (PBS containing 1% BSA, 0.05% NaN₃) for 2 h and washed with washing buffer (4 × 3 washes) and PBS. Cells were washed twice with FACS-buffer and incubated with PE-conjugated streptavidin (5 μg/ml) for 40 min at 4°C. Cells were washed twice with FACS-buffer and analyzed on a FACSCalibur cytofluorimeter (Becton Dickinson & Co.).

Upregulation of ICAM-1Expression in HUVEC

4 × 10^5 HUVEC were cultured in 25 cm² tissue culture flasks in M199 medium containing 20% FCS, 1% glutamine, 0.1 mg/ml ECGS, 0.1 mg/ml heparin for 16 h at 37°C. Medium was removed and cells were stimulated with either TNFα (10 ng/ml), htr-9 (10 μg/ml), utr-1 (10 μg/ml), or purified patient anti-TNFR55-IgG1 antibodies (10 μg/ml) for 6 h at 37°C in 2.5 ml medium. Cells were detached with trypsin/EDTA as previously reported [13], washed with FACS-buffer, transferrered to MTP (10^5 cells in 50 μl/well), mixed with 50 μl mouse monoclonal anti-ICAM-1 antibody in FACS-buffer and incubated for 30 min at 4°C. Cells were washed twice with FACS-buffer, incubated with PE-conjugated goat anti-mouse Ig for 30 min at 4°C, washed again twice with FACS-buffer, and analyzed on a FACSCalibur cytofluorimeter.

Neutralizing Activity of Anti-TNFR55-IgG1 Antibodies

ELISA. MTP were coated with 20 ng/well TNFR55-IgG1 in 100 μl 100 mM carbonate buffer, pH 9.6, overnight at 4°C. MTP were washed (4 ×) with 200 μl PBS, and unspecific binding sites were blocked with blocking buffer (2% dry milk powder in PBS, 0.02% Thimerosal) for 90 min at 37°C. MTP were rinsed with PBS, and either patient anti-TNFR55-IgG1 antibodies, htr-9, or utr-1 at various concentrations from 20 μg/ml to 20 ng/ml in 100 μl blocking buffer were added. MTP were incubated for 90 min at 37°C. After washing (4 ×) with PBS, containing 0.05% Tween 20 and addition of 40 ng/well HRP-conjugated TNFα in 100 μl blocking buffer, MTP were incubated for 90 min at 37°C, and washed (3 ×) with washing buffer and once with PBS. 100 μl peroxidase substrate was added. The reaction was stopped with 100 μl 2 M H₂SO₄ after 10–15 min and OD₄₅₄ was measured with a Dynatech MR5000 spectrophotometer.

Cellular assay. 10^4 KYM1-ID4 cells in 100 μl MEM10 culture medium/well were distributed in MTP. 50 μl of either patient anti-TNFR55-IgG1 antibodies or htr-9 were added (50 μg/ml in MEM10). Each well received 50 μl TNFα (60 ng/ml in MEM10), together with 50 μl of a 1:2 step dilution series of TNFR55-IgG1 from 2.5 μg/ml to 4.9 ng/ml in MEM10, and cells were cultured for 48 h. Supernatant was removed and crystal violet (0.5% in 70% methanol) was added for 1 min. MTP were rinsed several times with H₂O and allowed to dry completely. After addition of 150 μl/well 70% ethanol the plates were incubated for 10 min at RT on a lab shaker and OD₆₀₀ was measured with a Dynatech MR5000 spectrophotometer.

DOT Blot Assay

TNFR55-IgG1 (20 mg/ml) was denatured of in 1% SDS, 0.1% mercaptoethanol for 10 min at 95°C. Denatured and non-denatured TNFR55-IgG1 were spotted on nitrocellulose filters (100 ng/dot) using a dot blot apparatus (Bio-Rad). After washing with PBS, the filters were incubated with blocking buffer (2% dry milk powder in PBS, 0.02% Thimerosal) for 2 h at RT and cut into strips containing 4 parallel dots of either denatured or non-denatured TNFR55-IgG1. Strips were incubated with 500 μl of either biotinylated patient antibodies or biotinylated htr-9 (10 μg/ml in blocking buffer) overnight at RT. After washing with 500 μl blocking buffer (4 ×) strips were incubated with AP-conjugated streptavidin (1:5000 in blocking buffer) overnight (1:5000 in blocking buffer) for 2 h at RT. Strips were washed with blocking buffer (5 min, 3 ×) and PBS (5 min, 2 ×), and incubated with 250 μl AttoPhos (ECF-reagent) for 5 min at RT. Fluorescence was measured on a STORM 840 fluorimeter (extinction 450 nm; emission was detected using a 520 nm long pass filter) and signals were quantified using ImageQuant software.

Mapping of Linear Epitopes

Design of spots membranes. Arrays of 233 staggered 13-mer synthetic peptides covering the entire TNFR55-IgG1 sequence were covalently linked to cellulose membranes (Custom SPOTs service; Genosys Biotechnologies, Cambridgeshire, U.K.). The N-terminal amino acids of the 13-mer peptides were shifted within the TNFR55 and the IgG1 heavy chain sequence by two aa residues (aa #1 to #149, membrane M1 and aa #231 to #397, membrane M3), and by one aa residue across the fusion site (aa #151 to #230, membrane M2) (Fig 5a).
Binding of anti-TNFR55-IgG1 antibodies to immobilized peptides. SPOTs membranes were rinsed with methanol, washed twice with 15 ml PBS for 5 min and unspecified binding sites blocked with 15 ml blocking buffer (2 h at RT). Membranes were incubated in sealed plastic bags with 10 μg/ml patient anti-TNFR55-IgG1 antibodies, or htr-9, in 1.5 ml blocking buffer with/excess TNFR55-IgG1. (500 μg/ml) on a rotator (16 h at 4°C). Membranes were transferred into plastic containers and washed with 15 ml blocking buffer (5 min, 4°C). To reveal antibody binding, membranes were incubated with AP-conjugated goat anti-human Ig antibody [or AP-conjugated goat anti-mouse Ig antibody for htr-9] (1:5000 diluted in 10 ml blocking buffer, 2 h at RT). After washing with 15 ml blocking buffer (5 min, 3×) and PBS (5 min, 2×), 400 μl AttoPhos was applied to each membrane. After 5 min excess AttoPhos was removed, membranes were transferred into plastic bags and fluorescence was measured on STORM 840 fluorimeter [extinction 450 nm; emission was detected using a 520 nm long pass filter] (Fig 5b). The signals were quantified using ImageQuant software and were normalized against a standard curve that was determined in parallel using human IgG spotted in triplicates at various concentrations onto a nitrocellulose membrane. Thus, relative signal intensities of antibody reactivities to the peptide arrays were arbitrarily defined and normalized to this human IgG standard. All anti-TNFR55-IgG1 antibody studies were carried out with two identical sets of SPOTs membranes in parallel. Non-specific signals determined in presence of excess TNFR55-IgG1 were subtracted (Fig 5c).

RESULTS

Anti-TNFR55-IgG1 Antibodies in RA- and MS-patients in Double Antigen Assay

Plasma from RAiv- and MSiv-patients were obtained in blinded fashion from selected study centers, and thus included patients treated with placebo and active substance at various doses, whereas all RAiv-patients had received active drug. RAiv- and MSiv-patient plasma was first examined for TNFR55-IgG1 reactive antibodies with a double antigen assay using MTP coated with TNFR55-IgG1, diluted plasma and peroxidase-labeled TNFR55-IgG1 (Table 1). Signals were read against a parallel standard antibody assay. The double antigen assay format was highly sensitive, but prone to false positive signal especially at low titer; RF and other plasma components which cause also low positive signals in placebo treated patients contributed to this background which was significantly above the technical assay background (see footnote, Table 1). Large variations among individual patients on active drug were seen, with antibody concentrations from 0 to 23 EU/ml (mean: 3.0 EU/ml), and from 0 to 1.5 EU/ml (mean: 0.5 EU/ml), for RAiv- and MSiv-patients, respectively. These findings support earlier studies which had shown similar individual variation in magnitude and time of antibody formation in TNFR55-IgG1-treated RAiv-patients [6–12].

Semi-Quantitative Precipitation of Anti-TNFR55-IgG1 Antibodies

Antibodies binding TNFR55-IgG1 were precipitated from plasma of all three groups of patients using TNFR55-IgG1-coupled Sepharose-4B beads and separated into IgM and IgG class by SDS-PAGE. The gels

TABLE 1 Anti-TNFR55-IgG1 antibodies and RF in TNFR55-IgG1-treated patients

<table>
<thead>
<tr>
<th># of patients</th>
<th>Double antigen assay (EU/ml plasma)</th>
<th>IgG (μg/ml plasma)</th>
<th>IgM (μg/ml plasma)</th>
<th>RF (U/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAiv-patients: 200 mg 1)</td>
<td>38</td>
<td>3.0 (0–23)</td>
<td>5.5 (0–34)</td>
<td>27 (0–135)</td>
</tr>
<tr>
<td>RAiv-patients: Placebo</td>
<td>6</td>
<td>n.d.</td>
<td>0.9 (0–3.0)</td>
<td>3.1 (0–16)</td>
</tr>
<tr>
<td>5 mg</td>
<td>3</td>
<td>n.d.</td>
<td>1.2 (0.3–1.9)</td>
<td>3.7 (0.2–9.2)</td>
</tr>
<tr>
<td>12.5 mg</td>
<td>5</td>
<td>n.d.</td>
<td>1.9 (0–5.0)</td>
<td>5.6 (0–15)</td>
</tr>
<tr>
<td>25 mg</td>
<td>6</td>
<td>n.d.</td>
<td>4.0 (2.4–7.3)</td>
<td>19 (1.4–44)</td>
</tr>
<tr>
<td>50 mg</td>
<td>4</td>
<td>n.d.</td>
<td>16 (8.6–37)</td>
<td>10 (3.6–22)</td>
</tr>
<tr>
<td>MSiv-patients: Placebo</td>
<td>12</td>
<td>0</td>
<td>0.2 (0–1.0)</td>
<td>0.2 (0–1.0)</td>
</tr>
<tr>
<td>10 mg</td>
<td>11</td>
<td>0.5 (0–1.2)</td>
<td>0.6 (0–2.4)</td>
<td>0.9 (0–2.4)</td>
</tr>
<tr>
<td>50 mg</td>
<td>9</td>
<td>0.7 (0–1.5)</td>
<td>4.5 (0–13)</td>
<td>0.3 (0–1.4)</td>
</tr>
<tr>
<td>100 mg</td>
<td>8</td>
<td>0.5 (0–1.0)</td>
<td>5.2 (0–12)</td>
<td>1.4 (0–5.9)</td>
</tr>
</tbody>
</table>
| Data are means of all patients/group; the range is indicated in brackets. Data of the double antigen assay are indicated relative to a background of 0.5 equivalence units (EU)/ml detected in placebo treated patients; the technical assay background was 0.002 EU/ml. 1) total dose; dose schedule see methods section; 2) n.d. denotes not determined.
were stained with SYPRO-Orange fluorescent dye, protein was quantified by the emitted fluorescence at 570 nm against IgG and IgM standards on the same gel, and expressed as μg/ml plasma concentration (Table 1). In all three patient groups high individual variations were found consistent with the double antigen assay (Fig. 1A). In RAIV-patients, concentrations ranged from 0 to 34 μg/ml IgG (mean: 5.6 μg/ml), and from 0 to 135 μg/ml IgM (mean: 27 μg/ml). In RAsc-patients, concentrations through the different dose groups ranged from 0 to 37 μg/ml IgG (mean: 4.5 μg/ml) and from 0 to 44 μg/ml IgM (mean: 8.8 μg/ml). Despite the individual variations, the IgG response regularly increased with doses from 5 to 50 mg, whereas IgM was highest in the 25 mg dose group. Large differences in the IgM/IgG ratio were found. Rheumatoid factor (RF) in RAIV-patients strongly correlated with IgM (r = 0.93), but not with IgG concentrations (r = 0.63) (Fig. 1B). A similar positive correlation between RF and IgM, but not with IgG, was found in RAsc-patients (data not shown).

In MSIV-patients, the mean plasma concentrations of IgG were similar to those of RA-patients [3.0 μg/ml (range: 0 to 15 μg/ml), but the mean IgM concentrations were substantially lower [1.3 mg/ml (0 to 9 μg/ml)]. IgG, but not IgM concentrations increased with dose. Plasma RF was not detectable in most of the MSIV-patients in parallel to the absence or low content of IgM reactive with TNFR55-IgG1 (Fig. 1A).

Binding of Affinity Purified Anti-TNFR55-IgG1 Antibodies to Immobilized TNFR55-IgG1

For more detailed studies larger amounts of anti-TNFR55-IgG1 antibodies were affinity purified from plasma of selected patients. The binding of these antibodies to TNFR55-IgG1 was compared to that of the well characterized htr-9 and utr-1 mAb directed to TNFR55 and TNFR75, respectively [14]. The binding of the biotinylated patient antibodies to immobilized
TNFR55-IgG1 was studied by ELISA-based assays, using soluble TNFR55-IgG1 and human IgG for competitive inhibition (Fig. 2A). The titration curves of TNFR55-IgG1 revealed 50% inhibitory concentrations (IC50) of ~0.1 µg/ml and ~1 µg/ml for patient antibodies and htr-9, respectively. In contrast, human IgG had no blocking activity with either patient antibodies or htr-9 at concentrations up to 1 mg/ml (Fig. 2A). As expected, no significant binding of utr-1 to coated TNFR55-IgG1 was detected (data not shown).

**Binding of Anti-TNFR55-IgG1 Antibodies to Human Cells**

Purified anti-TNFR55-IgG1 antibodies of patients from all three groups were investigated for binding TNFR55 and potentially other components of human cell surfaces. The patient antibodies were biotinylated and binding to HL60 cells was studied by flow-cytometry using PE-conjugated streptavidin. All anti-TNFR55-IgG1 antibodies tested gave a positive cytofluorimetric signal; a representative example is shown in figure 2B. The mean fluorescence intensity was found to saturate between 1 and 10 µg/ml for both patient anti-TNFR55-IgG1 antibodies and htr-9. The maximum staining with anti-TNFR55-IgG1 antibodies consistently was substantially higher than with htr-9. A number of blocking studies were performed; whereas htr-9 staining was only inhibited by excess TNFR55-IgG1, the staining with anti-TNFR55-IgG1 antibodies could be competed by excess TNFR55-IgG1 as well as human IgG (Fig. 2B), indicating predominant binding to Fc-receptors rather than to cell-surface TNFR55. This conclusion was further supported by the inhibition of patient antibody binding to HL60 cells by anti-human CD64 mAb, but not by htr-9 (Fig. 2C).

**Upregulation of ICAM-1 Expression in HUVEC**

ICAM-1 expression in HUVEC cultures had been previously shown to be upregulated by selective TNFR55 activation by agonistic antibodies such as htr-1 and htr-9 [13]. This assay was used to investigate possible agonistic activities of the purified patient anti-TNFR55-IgG1 antibodies. Surface ICAM-1 expression was detected by flow cytometry. Stimulation with htr-9 led to an approximately 10-fold increase in specific surface ICAM-1 staining when compared to treatment with utr-1 used as negative control (Fig. 3A). The upregulation by htr-9 was slightly less pronounced than that seen with TNFα (10 ng/ml) in parallel studies; both activities were completely blocked by 500 µg/ml TNFR55-IgG1, but not by 500 µg/ml human IgG (data not shown). In contrast, no upregulation of ICAM-1 expression could be demonstrated with the anti-TNFR55-IgG1 antibodies of any of the RAiv- and MSiv-patients as shown by three representative examples (Fig. 3A). However, a 2–3 fold increase in ICAM-1 expression was detected with antibodies from 3 of 12 studied RAiv-patients as shown by one representative patient (#4817) in Fig. 3B. Intriguingly, an equivalent upregulation in ICAM-1 expression was also detected with antibody isolates prepared according to the identical protocol from the plasma of all three patients obtained at study entry, before start of TNFR55-IgG1 treatment. Furthermore, even though the anti-TNFR55-IgG1 antibody titers significantly changed through the treatment period (see figure 8A, below), the ICAM-1 upregulatory activity of those isolates did not change significantly with time (Fig. 3B). This activity could not be blocked by the addition of 500 µg/ml TNFR55-IgG1 indicating that it is independent of TNFα activity (data not shown); this excludes that the activity is due to TNFα which might have been co-purified with the patient antibodies. Artefactual stimulation of HUVEC due to the presence of LPS in the antibody isolates in concentrations high enough for activation could be excluded since LPS concentrations in the antibody isolates were <0.5 EU/ml and thus more than 1–2 orders of magnitude lower than those required for any measurable effect.
Anti-TNFR55-IgG1 Antibodies in RA and MS

A

B

C

log inhibitor conc. (μg/ml)

log fluorescence intensity

log fluorescence intensity

OD (490 nm)

log fluorescence intensity

log fluorescence intensity

Cell number

Cell number

Cell number

Cell number
Neutralizing Activity of Anti-TNFR55-IgG1 Antibodies

The interference of purified patient anti-TNFR55-IgG1 antibodies with the binding of human TNFα to TNFR55-IgG1 was investigated in ELISA-based and cellular assays. In the ELISA format, the neutralizing activities of patient antibodies and htr-9 were compared. Htr-9 neutralized TNFα binding to immobilized TNFR55-IgG1 in MTP with IC50 of 150 ng/ml. In contrast, no inhibition of TNFα binding could be observed with any of the patient antibodies tested in the same assay in concentrations up to 5 μg/ml, similar to the negative control data obtained with utr-1. The potential of patient anti-TNFR55-IgG1 antibodies to neutralize TNFR55-IgG1/TNFα interaction was further examined in KYM1-1D4 cytotoxicity assays, where protection by TNFR55-IgG1 against TNFα toxicity can be reversed by neutralizing antibodies such as htr-9. In contrast to htr-9, none of the tested anti-TNFR55-IgG1 antibodies of patients from all three groups, similar to utr-1 and buffer controls, had any neutralizing activity in this assay (see methods section for experimental detail).

Fc Receptor (FcR) Cross-linking by Soluble TNFR55-IgG1 and Anti-TNFR55-IgG1 Antibodies

Previous studies had shown that the binding of biotinylated TNFR55-IgG1 to HL60 cells was blocked by either TNFR55-IgG1, human IgG, or anti-human CD64 mAb but not by TNFα indicating predominant binding of TNFR55-IgG1 to Fc receptors [16]. The activation of monocytes via cross-linking of FcγRI has been reported to result in the release of inflammatory mediators such as IL-1β, IL-6, TNFα, and IFNγ [17–21]. To investigate whether patient anti-TNFR55-IgG1 antibodies could cross-link TNFR55-IgG1 bound to FcγRI and thereby stimulate cells, studies with THP-1 cells were carried out [16]. THP-1 cells were pretreated with IFNγ to induce FcγRI expression and cultured in MTP containing immobilized TNFR55-IgG1 or human IgG1 (coating range: 100 ng/ml to 100 μg/ml). Using IL-1β release as criterion, a coating density dependent cell activation was detected as revealed by an increase in IL-1β concentration in the 16 h cell culture supernatant in the range from 22 ± 3 to 178 ± 22 pg/ml and 25 ± 3 to 188 ± 3 pg/ml (mean ± SD, n = 3) for TNFR55-IgG1 and human IgG1, respectively, whereas no increase was detected with immobilized BSA [16]. However, no increase in IL-1β release was detected when soluble TNFR55-IgG1 and human IgG1 were used through a range of concentrations up to 10 μg/ml in the culture medium. The combination of soluble TNFR55-IgG1 (10 μg/ml) with purified anti-TNFR55-IgG1 antibodies (10 μg/ml) from patients of all three groups did not lead to any increased IL-1β production above the 25 pg/ml background value suggesting that cross-linking of FcγRI by TNFR55-IgG1 and anti-TNFR55-IgG1 antibodies did not occur, or did not lead to cell activation in this assay. In contrast, treatment of THP-1 cells with human IgG1 (10 μg/ml) and mouse anti-human IgG polyclonal antibody (10 μg/ml) in parallel control studies stimulated IL-1β production reaching 300 pg/ml culture supernatant [16].

Specific Recognition of Denatured and Non-Denatured TNFR55-IgG1

To investigate specificities for linear and conformational epitopes, denatured and non-denatured TNFR55-IgG1 was immobilized on nitrocellulose membranes and the
binding of purified biotinylated anti-TNFR55-IgG1 antibodies from selected patients was tested, using AP-conjugated streptavidin and ECF with AttoPhos as substrate (Fig. 4). The reactivity of antibodies to non-denatured TNFR55-IgG1 varied substantially in individual patients. To illustrate the relative amounts of antibodies reacting with denatured and non-denatured TNFR55-IgG1, presumably reflecting antibodies to linear and conformational epitopes, the signal obtained with non-denatured TNFR55-IgG1 was normalized to 100%. Comparing five selected patients from each group, antibodies to denatured TNFR55-IgG1 varied from 0 to more than 100%, suggesting that linear and conformational epitopes are recognized in a highly variable individual pattern.

**Mapping of Linear Epitopes on TNFR55-IgG1**

For mapping of linear epitopes arrays of 233 staggered 13-mer synthetic peptides covering the entire TNFR55-IgG1 sequence on SPOTs cellulose membranes were incubated with purified anti-TNFR55-IgG1 antibodies from selected patients of all three groups in presence or absence of excess TNFR55-IgG1 to determine specific signals (Fig. 5). With RAiv-patient antibodies, a core region of clustered peptides located in the TNF-receptor moiety in close proximity of the IgG1 joining site was identified (Fig. 6). One cluster of peptides with a core sequence of LPQIEN was recognized by antibodies from all but one tested RAiv-patients. This core sequence represents amino acids 167–172 of the TNFR55 sequence and is located 10 residues upstream of the fusion site between residues 182 and 183 of TNFR55-IgG1 [22]. The one patient non-responsive to the core sequence had only a very low total plasma anti-TNFR55-IgG1 antibody response in general and therefore served as negative control of the assay. The other linear epitopes in general were common to some, but not all RAiv-patients (Fig. 7). Interestingly, similar linear epitopes were recognized by antibodies from MSiv-patients (Fig. 6). As a consequence of the often low plasma concentrations of anti-TNFR55-IgG1 antibodies, epitope mapping could be performed in only a small number of MSiv-patients, but when possible, antibodies were also found reactive to the same cluster of peptides with the core sequence LPQIEN (Fig. 7). In contrast, antibodies from RAiv-patients recognized linear epitopes distinct from those recognized by RAiv- and MSiv-patient antibodies. With some RAiv-patients the antibodies did not react with any of the immobilized peptides (Fig. 6, right panel; Fig. 7). Other RAiv-patients carried antibodies with reactivities to other peptides not recognized by antibodies from RAiv- and MSiv-patients (Fig. 6, right panel; Fig. 7). Antibodies from some patients of all three groups reacted with peptides located in the hinge region of the IgG1-heavy chain fragment. These antibodies had an overall reactivity to the peptides #186–#190 with the CPAPELLLG core sequence which forms part of an TCPPCPAPELLGG-epitope recognized by anti-IgG auto-antibodies that occur naturally in healthy individuals [23, 24].
FIGURE 5  Mapping of linear epitopes. (A) Design of SPOTs membranes: Arrays of 233 staggered 13-mer synthetic peptides covering the entire TNFR55-IgG1 sequence with two-residue shifts (aa #1 to #149, membrane M1 and aa #231 to #397, membrane M3) and one-residue shifts (aa #151 to #230, membrane M2). (B) Example of reactivity pattern to immobilized peptides in a representative RA^-patient: 10 μg/ml patient anti-TNFR55-IgG1 antibodies in presence (right array) or absence (left array) of 500 μg/ml TNFR55-IgG1 were allowed to bind SPOTs membranes. Revealing with AP-conjugated goat anti-human Ig antibody and AttoPhos, and by chemiluminescent analysis. (C) Normalized specific signal intensities from figure 5B. The reactivity for each peptide is displayed as specific signal determined in absence and presence of excess TNFR55-IgG1 and normalized to a human IgG standard. Peptide numbering: The first residue of every tenth peptide is indicated.
Anti-TNFR55-IgG1 Antibody Response Throughout the Treatment Period

For three RA sc-patients (#4816, #4817, #6209) small amounts of plasma could be obtained at baseline and various time points throughout the treatment period and anti-TNFR55-IgG1 antibodies were affinity purified to reveal a time dependent profile of the antibody response and linear epitopes recognized. The data of two representative patients are shown in figure 8. Analysis by SDS-PAGE revealed for all three patients high amounts of IgM in the TNFR55-IgG1 reactive antibody fraction from day 0 (i.e. before start of treatment) and during the whole treatment period. In contrast, high IgG contents appeared in only two patients (#4816 and #4817) 28, 56, and 84 days after the first dose (Fig. 8A). Thus, whereas IgM class antibodies, presumably reflecting RF, were independent of TNFR55-IgG1 treatment, IgG class anti-TNFR55-IgG1 antibodies were first observed around day 28 and persisted to the end of the treatment period in these two patients. No further plasma was available from these patients after the end of treatment, but TNFR55-IgG1 reactive IgG concentrations were typically reduced to approximately 50% 6 months after the end of the treatment with RA sc-patients (data not shown).

Linear epitopes throughout treatment were mapped with the antibodies of two patients with low (#6209) and high (#4817) IgG concentrations at study end. At baseline patient #6209 had antibodies reactive with peptides of the IgG1-hinge region (Fig. 8B, chart a). This reactivity was detected in plasma at all times independent of TNFR55-IgG1 treatment. Throughout the entire treatment period other epitopes were not or only very weakly recognized (Fig. 8B, charts b through e). In contrast, no linear epitopes were detected with patient #4817 before the start of the treatment (Fig 8B, chart f). Antibodies with reactivity to linear epitopes appear between day 14 and 28 of treatment (Fig. 8B, charts g and h). This reactivity is restricted to the TNF-receptor moiety of the fusion protein and culminates in the recognition of two major linear epitopes located in close proximity to the joining region (peptides #162–#167) and near the N-terminus (peptides #29–#33) (Fig. 8B, charts i and j).

DISCUSSION

Here we report the formation of auto-reactive antibodies in RA and MS patients treated with recombinant human TNFR55-IgG1 in clinical studies. Despite wide individ-
ual variation, a major class of antibodies focussed on a cluster of linear epitopes located near its C-terminal end, i.e. in proximity of the TNFR55-IgG1 fusion site. The antibody fractions reactive with TNFR55-IgG1 included also RF and other pre-existing antibodies with specificities to the IgG1-moiety of the fusion protein. Interestingly, in contrast to the well characterized htr-9 antibody, the patient antibodies did not bind to and had no agonist activity at the 55kDa TNF receptor at the human cell surface. This might appear to be in conflict with the epitope mapping. An explanation is provided by the location of immunodominant linear epitopes near the transmembrane region of cell surface TNFR55 to which binding of an antibody would be sterically hindered at the cell surface. The unidentified co-purifying agonistic activities in the ICAM-1 assay detected in the plasma of some RA<sup>sc</sup>-patients surprisingly were also present at study entry and unchanged throughout the entire treatment period, and thus independent of TNFR55-IgG1 administration. The location of the major linear epitopes may also explain the apparent lack of neutralizing activity; protein crystallographic analysis has shown that the major TNFR55 interaction sites with TNF are restricted to the second and third receptor sub-domains [25] and thus are located N-terminal to the dominant epitope cluster. The reactivities of patient anti-TNFR55-IgG1 antibodies with denatured and non-denatured TNFR55-IgG1 presumably reflected specificities to linear and conformational epitopes. It is noteworthy that the reactivity of the anti-TNFR55-IgG1 antibodies from most RA<sup>iv</sup>- and RA<sup>sc</sup>-patients to denatured human IgG1 was completely abolished, whereas denatured recombinant soluble TNFR55 extracellular domain (i.e., the receptor moiety of TNFR55-IgG1) was still recognized, indicating that the conformational epitopes are preferentially located in the IgG1 moiety of the fusion protein (unpublished data). The lack of agonistic or neutralizing activities is consistent with the fact that no clinical

FIGURE 7  Clustering of linear TNFR55-IgG1 epitopes in SPOTs analysis. Linear epitopes recognized by anti-TNFR55-IgG1 antibodies of RA<sup>iv</sup>, MS<sup>iv</sup>, and RA<sup>sc</sup>-patients: Epitopes are indicated by the first residue of the first and the last of the staggered peptides recognized in each epitope (the amino acid core sequences of the individual epitopes are indicated in brackets). Reactivity of patients anti-TNFR55-IgG1 antibodies per epitope was scored positive if either one peptide generated a signal higher than a relative signal intensity (RSI) >100, or when more than one peptide reached RSI > 50 (black bar). Shifts of up to two amino acids among individual patient antibodies were accepted in some epitopes. 1) Anti-TNFR55-IgG1 antibodies of patient KA 5802 served as negative control. 2) Patients #4808 and #6206 were placebo treated.
FIGURE 8  Time dependence of anti-TNFR55-IgG1 antibody response (A) SDS-PAGE of anti-TNFR55-IgG1 antibodies precipitated with TNFR55-IgG1 coupled (+) or uncoupled (−) Sepharose 4B beads from plasma of two RA patients, #6209 (left panel) and #4817 (right panel), collected at study entry (before first dose) and days 1, 14, 28, 56, and 84 of treatment; IgG and IgM heavy chain reference bands are indicated. (B) Mapping of linear epitopes on SPOTs membranes recognized by patient anti-TNFR55-IgG1 antibodies from patients #6209 (a–e) and #4817 (f–j), collected at study entry (a and f), and days 14 (b and g), 28 (c and h), 56 (d and i), and 84 (e and j).
adverse events could be associated with anti-TNFR55-IgG1 antibody formation, even though the pharmacokinetic half-life was affected, the antibodies presenting an additional elimination pathway [6–11].

A novel sequence is generated at the fusion site of TNFR55-IgG1, since two unrelated human sequences are continuous at the fusion site. This potential T cell epitope may have generated an immunologically foreign element. However, the experience with IFNβ’s shows that such novel sequence elements are not necessary for an immune response to recombinant protein drugs to occur [26–28]; for example, the majority of IFNβ-treated MS-patients developed binding antibodies which reduced or abolished the normal biological and treatment effects [27]. Factors that influence such immune responses are likely to include glycosylation [28] and folding of the recombinant protein, dose, route and frequency of administration, and disease background and genetic susceptibility of the patients. Interestingly, the clinical treatment effect of TNFR55-IgG1 did not correlate with antibody formation; although both RA- and MS-patients formed antibodies, a beneficial effect was seen in RA- [6–11] and a negative in MS-patients (Arnason, B. G. W. et al., for The Lenercept Multiple Sclerosis Study and The University of British Columbia MS/MRI Analysis Groups, in preparation).

Our studies provide insight in two further issues of the human immune response. First, immunodominance of epitopes within a given protein is thought to be mainly influenced by surface accessibility [29, 30]. The major linear epitope (LPQIEN) recognized by RAα- and MSα-patients was found to be located between aa 167 and aa 172 of the TNFR55 sequence immediately N-terminal to the IgG1-hinge fusion site. Protein crystallography studies of the TNFR55 extracellular domain support that its C-terminal end presents a disordered structure [25, 31]. Joining the TNFR55 C-terminus to the hinge of human IgG1 generated a stretch of amino acids which may be seen as continuous by the immune system. A similar finding has been reported in chronic myelogenous leukemia where a chromosomal translocation results in the generation of a p210BCR-ABL chimeric protein with abnormal tyrosine kinase activity [32]; the BCR-ABL joining region peptides formed new epitope(s) and elicited a specific (auto-) immune response [32]. The second issue concerns epitope spreading which has been repeatedly demonstrated in murine models of autoimmune diseases [33, 34]. In our study, time-dependent variation of linear epitopes could be observed only in one patient (patient #4817, RAα). Interestingly, the linear epitopes concentrated on two major regions, one coinciding with the dominant cluster, at the end of the treatment. These findings support the general conclusion that remarkable consensus patterns emerged in the anti-body response despite the heterogeneities in the patient populations.

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